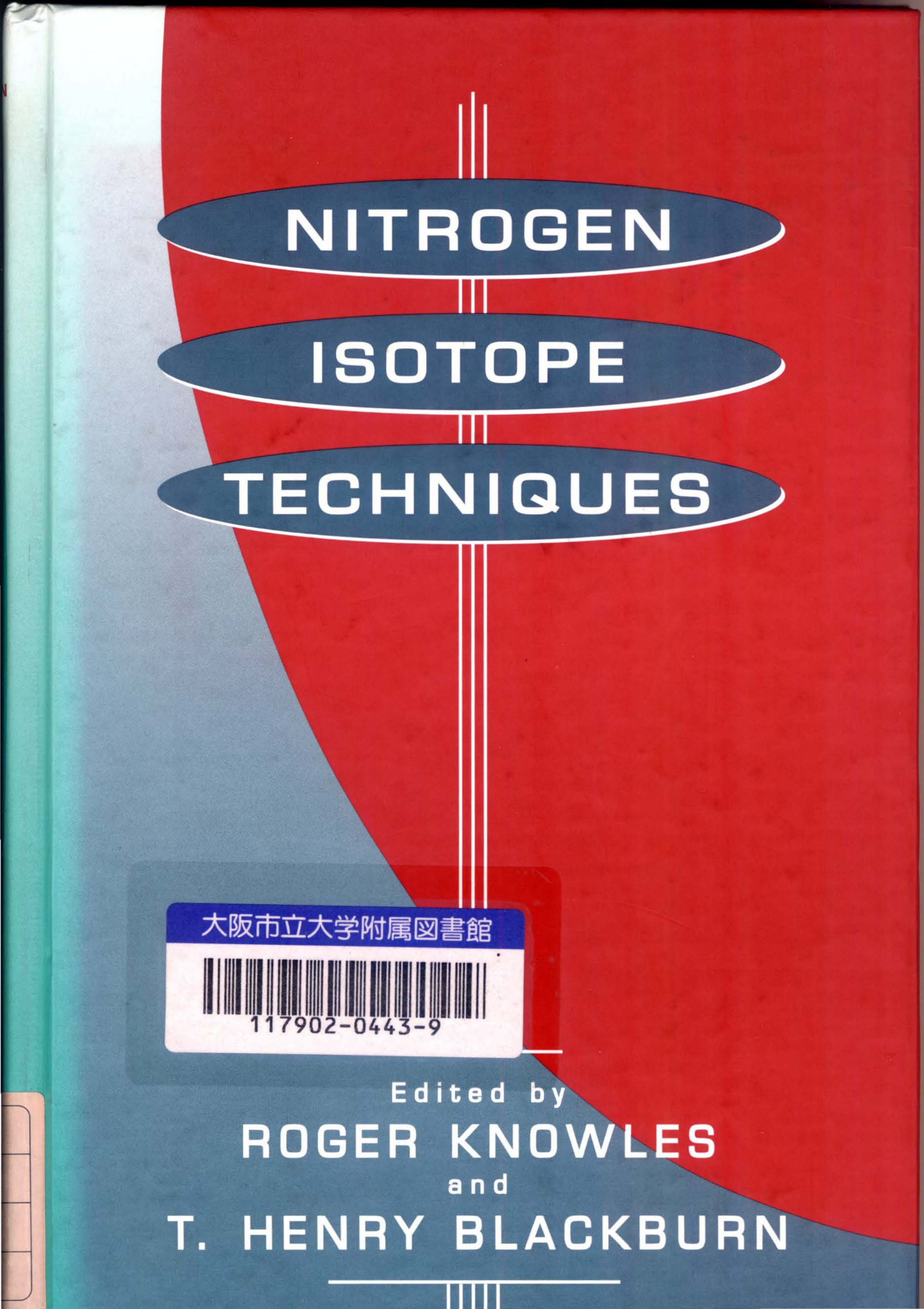

NITROGEN ISOTOPE TECHNIQUES

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Preface

Several books dealing with the use of nitrogen (N) isotopes were published during the last 10 years. They covered separately areas such as soil studies, nitrogen fixation, marine systems, and other topics. To our knowledge, this is the first volume to bring together basic aspects of the measurement of the stable and radioactive isotopes of N as well as specific applications in plant, soil, and aquatic biology. This is the second volume in the series *Isotopic Techniques in Plant, Soil, and Aquatic Biology,* published by Academic Press.

Nitrogen Isotope Techniques and other volumes in the series provide reference sources from which detailed methods can be found for isotopic studies, in our case, nitrogen isotopes. The descriptions of methods are sufficiently detailed, so it should be possible to follow procedures without referring to other sources or the primary literature. However, key sources are given to permit such referencing if necessary. The volume should be of particular interest to researchers and students engaged in physiological or ecological studies of natural and agricultural systems. We hope that it may be treated by such persons as a laboratory cookbook.

After an introductory chapter, there follow separate chapters on basic methods for mass spectrometry and emission spectrometry, the two major techniques for the measurement of ^{15}N abundance. Discussion and description of sample preparation, with some introduction to automated methods, is also included. Chapter 4 describes some of the special approaches and techniques necessary when precise determination of ^{15}N natural abundance is desired, and calculation methods are described. Specific calculations are also included when relevant to other chapters.

Nitrogen fixation measurements employing both enriched ^{15}N-dinitrogen and so-called isotope dilution after addition of enriched inorganic-^{15}N are covered in Chapters 5 and 6. Chapter 5 deals with applications in terrestrial (dryland) plant–soil systems, and Chapter 6 deals with aquatic and wetland systems, including the highly important rice paddy. Applications for nitrification and dentrification (the production and reduction, respectively, of oxides of nitrogen) are covered in Chapter 7. Next, Chapters 8 and 9 address terrestrial and aquatic applications in the field of assimilation, mineralization, and turnover of organic nitrogen. Finally, Chapter 10 describes the very specific methods required for the proper use of the radioactive isotope ^{13}N.

We hope that the methods described allow researchers to adapt the procedures to virtually any task encountered in the investigation of nitrogen transformation processes.

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1

Introduction

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The purpose of this introduction is to give an overview of how nitrogen isotope analysis has been used in different ecosystems, how useful this methodology has been, and to give some assessment as to possible future developments and lines of research. The views expressed here are personal and do not necessarily reflect those of the authors of the different chapters, whose forgiveness we beg should they feel that we have undermined their statements. We do not refer to specific chapters and we do not cite specific references.

In general, nitrogen isotope analysis has been useful in the routine measurement of transformation rates of nitrogen-containing compounds in agricultural but less so in natural ecosystems. The employment of these isotopes has, however, been very helpful in elucidating specific pathways and demonstrating that certain reactions are possible. As the cost of mass spectrometers falls and they become easier to use, the use of nitrogen isotopes will likely become more common in future routine measurements, but inherent difficulties remain, and they may be impossible to overcome. Some of these difficulties will be indicated in the context of the application of isotopes to measure specific processes in the nitrogen cycle. The main problem is that of adding trace amounts of label (^{15}N) to very small and reactive pools of target compounds, where reaction rates would be influenced by an increase in substrate concentration. In the future, it is anticipated that increased experimental ingenuity will overcome some of

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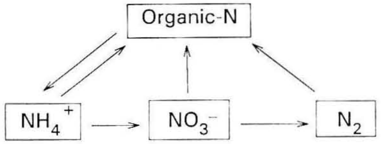

Figure 1 Components and interactions in the nitrogen cycle.

these technical difficulties. We expect to see increased use of high-pressure liquid chromatography and gas chromatography in the preparation of samples for analysis and increased use of the direct analysis of ^{15}N compounds, rather than the conversion of all nitrogen to N_2, before measurement by mass spectrometry. There are very good reasons for using N_2 for mass spectrometry analysis, but its exclusive use may have resulted in a restriction of experimental design.

The most important processes in the nitrogen cycle are outlined in Fig. 1. They have been described in the various chapters in relation to the ecosystems found in water, sediment, and soil. The processes are organic-N mineralization to NH_4^+; organic-N synthesis from inorganic NH_4^+, NO_3^-, and N_2; the oxidation of NH_4^+ to NO_3^- (nitrification); and the reduction of NO_3^- to N_2 (denitrification). We will restrict our observations to the microbiological aspects of the nitrogen cycle; it is the end-products of the microbial reactions that are available for plant uptake and other processes. The mineralization (not remineralization) of organic matter occurs via a number of hydrolytic, fermentative, and oxidative processes to simple C1 compounds and to NH_4^+, irrespective of whether the degradation occurs in oxic or anoxic conditions. Surprisingly little is known about the specific microorganisms involved in the various degradative steps: There are probably as many different types as there are organic compounds. The point of interest is that NH_4^+ is the exclusive end-product of nitrogen mineralization. The synthesis of organic N (biomass) is also carried out by a very diverse group of microbes, probably just as diverse as the degrading microorganisms and may often be the same microorganisms, in many situations. These are heterotrophic microorganisms; they are almost always energy-limited, and, as a result, the metabolic pattern is to utilize the energy in the reduced carbon of an organic molecule, the nitrogen being excreted as NH_4^+. The NH_4^+ will subsequently be taken up and built into biomass. This is the pattern observed in the metabolism of amino acids: deamination, carbon oxidation, and NH_4^+ reincorporation. There is always some direct incorporation of the amino acid directly into biomass, but the proportion is probably small. The order of preference for incorporation is NH_4^+, NO_3^-, and N_2. All but the most fastidious bacteria can utilize NH_4^+ and many aerobes can use NO_3^-, but only a restricted group of bacteria can utilize N_2. These nitrogen-fixing bacteria may be aerobic or anaerobic and heterotrophic or autotrophic, and the latter may be phototrophic or chemotrophic. All suffer from the same restriction; nitrogen fixation requires the expenditure of much energy and reducing power, and the enzymes are O_2-sensitive. As a result, the process is essentially anaerobic and very often associated directly or indirectly with photosynthesis, where energy is less limiting.

The oxidative step in the nitrogen cycle is the reaction of NH_4^+ with O_2 to give NO_3^- via a number of intermediate molecules. The first step is to NO_2^- by a specialized group of chemoautotrophic bacteria, whose biomass is low but of wide distribution. Nitrite seldom accumulates but is oxidized further to NO_3^-, again by a group of specialized bacteria, most of which are chemoautotrophic. Some heterotrophic nitrification occurs but is thought to be of limited importance. The main significance of nitrification is that it leads to the possibility of leaching losses and of denitrification. This results in the conversion of a readily utilizable compound (NO_3^-) to one that cannot be assimilated by most bacteria or by any eukaryote. Globally, denitrification is balanced by nitrogen fixation, but locally the processes are seldom of equal magnitude. Denitrifying bacteria are generally aerobic heterotrophs, which in an anoxic environment have the capacity to utilize NO_3^- instead of O_2 as an electron sink in the oxidation of reduced carbon or in restricted cases of H_2S.

Ideally, it is desirable to describe an ecosystem's nitrogen cycle in relation to the rates of appearance and disappearance of all the pools, as shown in Fig. 1. In addition, it is helpful to know something about the fate of carbon, its rate of oxidation, and the rate of biomass-C synthesis, thus linking the C and N cycles. In many situations, it is not simply the cycling of C and N between the microbial compartments that is of major interest; it is the availability of N for plant uptake that is of paramount importance. To some extent, the availability of N for assimilation by plant roots may be determined from the net production of inorganic N (NH_4^+ and NO_3^-) in the system, on the assumption that this would be available to the plant. There are some dangers in this approach because the presence of roots will almost certainly affect the O_2 balance in the system and, thus, affect the coupled processes of nitrification and denitrification.

Water, sediment, and soil are the three basic environments, and these have been selected for treatment in the individual chapters. This reflects more the conventions, which are maintained by tradition and scientific institutions (universities and government agencies), than any real basic differences among them, at least in relation to the microbial process involved. Microorganisms can only grow and express their metabolism in water: The three ecosystems are essentially aqueous. The difference between them lies in the quantity of water present, the quantity of organic input, and the accessibility of O_2. All three factors are interrelated. The amount of water will determine whether O_2 penetration is restricted to diffusion through water or through air, the latter process being significantly more rapid. The quantity of organic input will also determine the depth of O_2 penetration, because O_2 will be consumed by organic-C oxidation. The quantity of organic input will also determine the availability of NH_4^+ to fuel the other N-cycle processes. The only real difference between saltwater and freshwater lies in the high SO_4^{2-} content of the former, which leads to a complete oxidation of organic carbon with the production of H_2S in anoxic marine sediments. In anoxic freshwater sediments, the products of carbon metabolism are CO_2 and CH_4. Oxic systems (soil, mixed waters, organic-deficient sediment, and sediments with rooted plants) are characterized by the presence of NO_3^- as the main inorganic nitrogen compound: Most NH_4^+ is oxidized, the NH_4^+ concentration is low, and the NH_4^+ pool turns over rapidly. In contrast, anoxic systems (water-saturated soils, organic-rich sediments, and stratified waters) contain only the NO_3^- that has been imported; NH_4^+ concentrations are high and this large pool turns over relatively slowly. These facts have considerable significance in the strategy of ^{15}N tracer methodology. Most interest lies, not so much in the completely oxic or the completely anoxic ecosystems, but in the interface between these systems. It is at the interfaces that the most important reactions occur. Ammonium can diffuse or be transported across the interface, from anoxic to oxic, where it may be transformed to NO_3^-. The NO_3^- can then diffuse back to the anoxic and be denitrified. Similarly, O_2 can diffuse into the anoxic regions and lead to similar reactions. Oxic–anoxic interfaces can be extensive and well defined, as in marine and lake sediments and the surface of marshes and bogs. They can also be less well defined and less extensive, as around plant roots and animal burrows in anoxic soils and sediments or in microzones surrounding decomposing organic particles in any of the oxic environments.

The question arises as to why this book is organized in a conventional manner, based on the mutually exclusive water–soil–sediment ecosystems, when it might seem rational to have attempted a more fundamental synthesis, based on oxygen–water–organic relationships. There are several answers to this question, none of which is entirely satisfactory. Most researchers who work with nitrogen isotopes and who will use this book belong to one of the traditional disciplines and might not find a nontraditional format as useful. In other words, they might not buy the book. We cannot complain that the authors have not fulfilled the task assigned to them; they have faithfully restricted themselves to their assigned topic. It is remarkable, however, how seldom cross-references occur to other ecosystems. It is our hope that the present reader will be less restrictive and will bravely cross the boundaries of tradition and learn that the same processes occur in different systems and that the isotope methods developed for one ecosystem can be profitably used in another. It is for this reason that in this brief survey, we concentrate on the use of labeled substrates, because they may be applied in any environment. First, however, a brief word on the isotopes, their measurement, and their natural abundance.

The N-isotopes are stable ^{15}N and radioactive ^{13}N. Use of the latter has been restricted to very few laboratories. The half-life is very short, the isotope must be produced close to the experimental site, and the methods for analysis of processes and products are very specialized. It must be emphasized that the use of ^{13}N has led to the elucidation of processes that could not otherwise have been investigated. It can be added to label small active pools to determine their turnover; this would be impossible with ^{15}N tracer methodology. We are, however, mostly concerned with ^{15}N, because this is the N isotope that is commonly used. The methodology for measurement of ^{15}N is described in considerable detail in the following chapters. We may be criticized because too many chapters contain descriptions for isotope measurement. It is hoped that this will not be a cause of annoyance to the reader; we thought that it was better to have some overlap rather than to have chapters that were not self-contained. The two main methods for N isotope analysis are emission spectrometry and mass spectrometry. The former, in the past, has not required such large samples as the latter and, for that reason, has been useful in many situations. The $^{15}N/^{14}N$ ratio is determined in N_2, usually prepared by a Dumas oxidation of NH_4^+ or organic N. Newer mass spectrometers require smaller sample sizes for accurate analysis, and better methods allow for the preparation of samples from materials that contain low concentrations of nitrogen. It is usual, if not universal, to measure $^{15}N/^{14}N$ ratios in N_2 in mass spectrometers. Modification of mass spectrometers, particularly in the method of ionization, should allow for more flexibility in the N compounds that are analyzed for ^{15}N content. It is difficult to predict what these compounds might be, but one might anticipate the use of ammonium derivatives, organic compounds resulting from NO_3^- derivatization, and amines. In the past there seems to have been some lack of a spirit of adventure in exploring the analysis of complex organic compounds in favor of the safe, but not always relevant, quest for ever greater precision in isotopic analysis of N_2.

The analysis of natural abundance $^{15}N/^{14}N$ ratios requires considerable precision, because there is not much evidence for large changes in these ratios in natural pools of nitrogen. This is equivalent to deducing that biological processes do not discriminate actively between molecules containing the light— as opposed to the heavy—isotope. Considerable discrimination occurs during denitrification, probably some during nitrogen fixation, and interesting changes during organic-N mineralization. There is an enormous attraction in being able to use the natural $^{15}N/^{14}N$ ratios to deduce the rate of turnover of, or the source of input to, a particular N pool. The method has great potential, many researchers are working with various aspects of individual processes and food chain transformation, and, hopefully, future developments will fulfill the great expectations of so many laboratories. It is our assessment that results to date have been disappointing.

The analysis of $^{15}N/^{14}N$ ratios after the addition of tracer ^{15}N also requires precision, but often the limiting factor is the low concentration of the particular pool being investigated and the difficulty of obtaining enough sample for analysis. This problem will recur frequently as we consider the use of ^{15}N-labeled NH_4^+, NO_3^-, N_2, and organic N to analyze processes in the N cycle. There are three main experimental approaches: The dilution of the label in the pool may be measured to give the rate of production, or the rate of disappearance from the pool may be measured to give a rate of consumption. The rate of appearance of label in another pool gives the rate of production of that compound.

$^{15}NH_4^+$ *as Tracer*

Three arrows connect NH_4^+ with other pools (Fig. 1), but, in addition, labeled $^{15}NH_4^+$ can be used to see the exchange between dissolved-, exchangeable-, and bound-NH_4^+ pools, in soils and sediments. Possibly the most important use of $^{15}NH_4^+$ is in the measurement of the rate of organic-N mineralization. Ammonium is the final product of organic-N mineralization in all environments, and it is assumed that this NH_4^+ has the normal $^{15}N/^{14}N$ ratio (this remains to be proven). This NH_4^+ dilutes the added $^{15}NH_4^+$ and the rate of dilution reflects the rate of organic-N mineralization. Any disappearance of the $^{15}NH_4^+$, due to uptake or oxidation, and any change in pool size during the experiment can be taken into account in the calculation of the true rate of dilution. It should be remembered that oxic and anoxic ecosystems differ in the size and turnover rate of the NH_4^+ pool. There are real problems in studying tracer dilution when the concentration of NH_4^+ is very low, as in oligotrophic waters. First, it is impossible to add the label at concentrations approaching tracer levels (10%) when the *in situ* concentration may be only 0.5 μM and the rate of NH_4^+ uptake by algal cells is high. Second, processing liters of seawater to get enough sample for analysis is not simple. Some ingenious solutions to the latter problem have been used, and these are described in the relevant chapters. One solution to the adding of label is to make the assumption that the concentration of added NH_4^+, the end-product of organic-N degradation, does not affect the rate of degradation. There would, therefore, be no disadvantage in adding excess amounts of label. This assumption is very likely true for most situations, where the heterotrophic microorganisms, which are responsible for the mineralization of organic N, are probably energy-limited and not stimulated by the addition of excess NH_4^+. This may not always be true, especially if the substrate(s) for the heterotrophic microbes has a high C : N ratio. This could be the situation in pelagic environments, where the organic substrate used by the heterotrophs might be largely carbohydrate, released by the photosynthetic phytoplankton. Similarly, heterotrophic microorganisms in the oxic rhizosphere may largely depend for energy on organic molecules of low nitrogen content, which have been secreted from the roots. It is unlikely that the addition of large amounts of $^{15}NH_4^+$ to anoxic environments can have any effect on the rate of NH_4^+ production by the heterotrophic population. The *in situ* NH_4^+ pool is usually high in anaerobic sediments, soils, and waters: NH_4^+ is not a limiting nutrient. The problem in these environments is not that of label addition or of obtaining sufficient sample for analysis; it is a problem of a slow rate of NH_4^+ production, which gives a slow rate of $^{15}NH_4^+$ dilution. Usually the gross rate of NH_4^+ production is of little interest, particularly in this type of environment. The net rate of production is of most interest, because this is the rate that determines the amount of NH_4^+ that will be free to be transported to an oxic interface, where it can enter into a more general nitrogen cycle. The measurement of net rates of production does not depend on nitrogen isotope technology.

The second rate that can be measured, following the addition of $^{15}NH_4^+$ to a natural pool, is that of incorporation into biomass. In situations where uptake and incorporation into biomass is the only mechanism for the disappearance of label (taking account of any changes in pool size), the rate of disappearance may be used to calculate the rate of incorporation of NH_4^+ into biomass. Pelagic systems are an example of this type of environment, where the rate of nitrification is insignificant. In this, as in all other systems, there is an incorporation of label into the heterotrophic population, and it is only the surplus NH_4^+ that is available to the algal population. The synthesis of biomass, calculated from $^{15}NH_4^+$ disappearance, may, therefore, be higher than that obtained by measuring directly the amount of ^{15}N that had been incorporated into phytoplankton cells. This latter method is more direct and has been used for many years to measure rates of incorporation into pelagic phytoplankton. The directness of the method is attractive, but there is a penalty to pay. The rate of NH_4^+ uptake is concentration-dependent, and the problem again arises of adding sufficient label to a very small pool so that the uptake of this label may be detected. This must be achieved without altering uptake rates. It is not surprising that the more indirect method is increasingly used in this type of oxic environment. Measurement of $^{15}NH_4^+$ incorporation into biomass in anoxic environments is complicated by the large NH_4^+ pools, the slow rates of incorporation, and a lack of interest in the rate obtained. The uptake rate can be measured either from $^{15}NH_4^+$ disappearance or from appearance in biomass. The latter measurement is complicated by the very large background of organic N, which is usually present in, for example, anoxic sediments and soils. It is likely that future developments will include methods for the selective extraction of newly synthesized organic N (proteins, polynucleotides), in which the ^{15}N content can be measured. It is not true that there is a total lack of interest in biomass synthesis in anoxic environments. The incorporation of $^{15}NH_4^+$ is one of the few methods for assessing microbial growth in these ecosystems.

Ammonium exists in multiple pools in both oxic soils and anoxic sediments. The NH_4^+ dissolved in pore water is in equilibrium with exchangeable NH_4^+, which is loosely bound to particles. There is a third pool associated with clay mineral lattices, which is in very slow equilibrium with the other two pools. The exchange of NH_4^+ between these pools has been studied by observing the rate of transfer of added $^{15}NH_4^+$ from pore water to the sediment particle pools. It should be noted that these processes have significance in experiments where $^{15}NH_4^+$ is added to soil and sediment pools, to study biological rates. The physical factors that determine NH_4^+ distribution must also be recognized.

The third flux, which involves NH_4^+ (Fig. 1), is the loss to NO_3^-. This process of nitrification is restricted to oxic environments (soils, waters, and the superficial layers of sediments). Although some nitrification occurs in pelagic waters, the rates are low in relation to the other fluxes, which involve NH_4^+ in these waters, and are relatively uninteresting. The rates of nitrification in oxic soils are high: Virtually all the net production of NH_4^+ is rapidly oxidized to NO_3^-. Rates of nitrification are thus equivalent to rates of NH_4^+ production. The most interesting situation in which nitrification occurs is at the oxic–anoxic interface of sediments, roots, worm burrows, etc. It is at these interfaces that coupled nitrification–denitrification occurs, and it is here that the rate of nitrification is most difficult to measure. Nitrification occurs in a very narrow zone at this interface, possibly the zone is of millimeter dimensions, at least in situations where nitrification rates are high. There is thus the technical difficulty of adding $^{15}NH_4^+$ to this layer in tracer amounts, of measuring the specific activity of this NH_4^+ pool, and finally of measuring the amount of ^{15}N label that appears in the very small NO_3^- pool. The concentration of the latter may not exceed 10 μM, ~1 nmol cm^{-2}, or 10 μmol m^{-2}. It might thus require the extraction of the NO_3^- pool from 1 m^2 of sediment for one ^{15}N determination. While sample size requirement is likely to decrease and extraction methods are expected to improve, there remains the problem of determining the $^{15}N/^{14}N$ ratio in the small and highly dynamic NH_4^+ pool, at the oxic interface. Considerable ingenuity has been demonstrated in the construction of continuous flow chambers, where labeled NH_4^+ can be supplied in the perfusing water. These systems solve some, but not all, of the difficulties involved in measuring rates of nitrification. At present, nitrification can probably be better measured by conventional methods, which do not involve ^{15}N.

$^{15}NO_3^-$ *as Tracer*

The dilution of added $^{15}NO_3^-$, in all environments, gives the rate of nitrification. The comments in the preceding paragraph are equally relevant, in this context. The situation where it is of most importance to measure the rate of nitrification is also the most difficult. Labeled NO_3^- can be added to sediment or to overlying water in a flow-through system, but in neither situation can the rate of dilution of the sediment pool be measured unambiguously.

The contribution of NO_3^- to the increase in microbial or algal biomass N can be calculated from the appearance of ^{15}N label from NO_3^- in cells. This is of little interest in sediments but is of considerable interest in determining the uptake of NO_3^- by pelagic phytoplankton. It is mostly in this context in which rates of incorporation have been measured. The rate of nitrification is insignificant in this system, so no dilution of added label occurs. It is important that tracer quantities of $^{15}NO_3^-$ be added, so that uptake rates are not stimulated. In many situations it would seem that measurement of NO_3^- disappearance might be a more appropriate methodology. The addition of $^{15}NO_3^-$ to soils has largely been in the context of measuring the rate of incorporation of ^{15}N label into plants. It is important to measure and make allowance for any changes in the $^{15}N/^{14}N$ ratio of the NO_3^- that might occur during the experimental period.

The most significant use for $^{15}NO_3^-$ is in the measurement of rates of denitrification. The appearance of label in $^{15}N_2$ would be related to the rate of reduction of $^{15}NO_3^-$. Flow-through systems with marine sediments, containing $^{15}NO_3^-$ in the perfusing water, allow for equilibrium conditions to be established and for a good measurement to be made of the rate of denitrification from externally supplied NO_3^- . Unfortunately, this experimental procedure does not measure denitrification that depends on internally produced NO_3^-. It seems very likely that future developments will allow for better measurements of coupled nitrification–denitrification rates in sediments. Sadly, this book lacks a chapter on sediment denitrification.

The absence may not be so serious at this time, because it seems that this most important potential use of $^{15}NO_3^-$ has not yet been fully realized. The problems associated with the narrow oxic–anoxic interface in sediment are not relevant in the measurement of denitrification rates in soils. There are usually large NO_3^- pools in soils, which can be labeled with $^{15}NO_3^-$, and the rate of production of $^{15}N_2$ may be relatively easily measured. Denitrification is a slow and quantitatively small component of N cycling in pelagic systems.

$^{15}N_2$ as Tracer

Potentially, $^{15}N_2$ dilution could be used to measure the rate of denitrification, but it is never employed in this context. Among other problems would be the difficulty of obtaining a random distribution of label in the N_2 in soils and sediments, the sites of most interest in denitrification studies.

The main use for $^{15}N_2$ has been in the direct measurement of ^{15}N incorporation into nitrogen-fixing populations. This has seldom been used on a routine basis, but it has been very useful in establishing the validity of acetylene reduction measurements, particularly for pure cultures. Nitrogen fixation is inhibited by the presence of fixed nitrogen and is thus of most importance in ecosystems where nitrogen is limiting. This is often true for soils from which plants have depleted the available nitrogen. It is also true for many aqueous, particularly marine, systems, and photosynthetic cyanobacteria are the most significant contributors to nitrogen fixation in these environments.

^{15}N-Organic Compounds as Tracers

The use of ^{15}N-organic compounds as tracers has been neglected, partially because of the difficulty of obtaining labeled organic molecules and because the rate of organic-N mineralization can be measured by NH_4^+ production. Labeled urea has been used to determine rates of uptake by phytoplankton and to investigate mutual interference in the uptake of urea, NH_4^+, and NO_3^-. Limited use has been made of ^{15}N-labeled amino acids. It seems unlikely that the use of ^{15}N-labeled organic molecules will become routine, but it is predicted that for specific applications, they will prove to be very useful.

Our final conclusion is that ^{15}N methodology has contributed greatly in process-based studies and that it probably will soon contribute to the advance of our knowledge of N cycling at the ecosystem level.

2

Mass Spectrometry

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I. INTRODUCTION

Although other techniques may be employed for N isotope analysis, isotope ratio mass spectrometry is the method of choice for quantitative determinations because it provides unmatched precision. This is no small advantage, in that the sensitivity achieved in detecting tracer N is ultimately determined by analytical precision, and any improvement in precision translates into a gain in sensitivity. As a result, use of mass spectrometry for N isotope analysis has the advantage over other techniques that lower concentrations of tracer N can be detected, which extends the scope of N-tracer research and improves detection of treatment effects. Moreover, ^{15}N-depleted materials may be utilized, and measurements can be made of variations in the natural abundance of isotopic N.

The mass spectrometer is, unfortunately, a complicated and expensive instrument that requires considerable care to operate and maintain, and this has been a major limitation in the use of N isotope techniques. However, the technical difficulties have been reduced substantially by advances in the design of mass spectrometers during the past two decades, due largely to utilization of solid-state electronics. The greatest advance has undoubtedly been the development of the automated mass spectrometer, which can perform isotope ratio analyses on microgram quantities of N, at a rate of up to several hundred samples per day. As a result, many of the analytical problems traditionally associated with N isotope research have been elimi-

NITROGEN ISOTOPE TECHNIQUES
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nated, particularly for those who submit samples to a laboratory offering N isotope analyses as a service.

Information about the procedures used in N isotope analysis by mass spectrometry can be found in a number of excellent publications, including those by Bremner (1965d), Fiedler and Proksch (1975), Hauck and Bremner (1976), Edwards (1978), Bergersen (1980), Buresh *et al.* (1982), Hauck (1982), Haystead (1983), Fiedler (1984), and Robinson and Smith (1991). In the present treatment, consideration is given to the preparation of samples for analyses by automated mass spectrometers as well as to Kjeldahl–Rittenberg analyses using manually operated instruments. Consideration is also given to basic aspects of mass spectrometry and recent advances in automation, but no attempt is made to evaluate the various instruments that are commercially available.

II. INSTRUMENTATION FOR N ISOTOPE ANALYSIS

A. Principles of Operation

There is considerable variety in the design of mass spectrometers, but the great majority of instruments used for N isotope analysis are single-focusing, magnetic deflection spectrometers with an electron impact ion source, and the analysis is invariably carried out with N in the form of dinitrogen (N_2). Operation of such an instrument during isotope ratio analysis of N_2 is illustrated in Fig. 1, which shows a schematic diagram of the analyzer assembly of a 60°-sector mass spectrometer equipped with double collectors. The analyzer consists essentially of three components: the analyzer tube, the ion source, and the collectors. The analyzer tube is fabricated from stainless steel or an alloy (often Inconel®), with its center flattened laterally and curved through an angle of 60° (other designs use an angle of 90 or 180°). The ion source and collectors are mounted at opposite ends of the analyzer tube via flanges sealed by gold wire O-rings.

From a suitable inlet system (see Section II.D), N_2 is admitted to the ion source through a leak (a constriction in the inlet line that determines the flow rate) for bombardment by electrons from a heated filament. The electron beam is accelerated across the incoming flow of N_2 molecules by a positive potential (typically 70 volts) between the filament and an anode (commonly referred to as a trap), such that the energy of the electrons greatly exceeds the ionization potential of N_2. Ionization results largely from the loss of a single electron to form $^{28}N_2^+$, $^{29}N_2^+$, and $^{30}N_2^+$. To minimize ion–molecule reactions, pressure in the ion source is maintained at <1 mPa by the use of a high vacuum pump (see Section II.C), which operates continuously. Nevertheless, the efficiency of ionization is quite low (typically of the order of 0.1%), and most of the molecules that enter the ion source are removed by the vacuum pump.

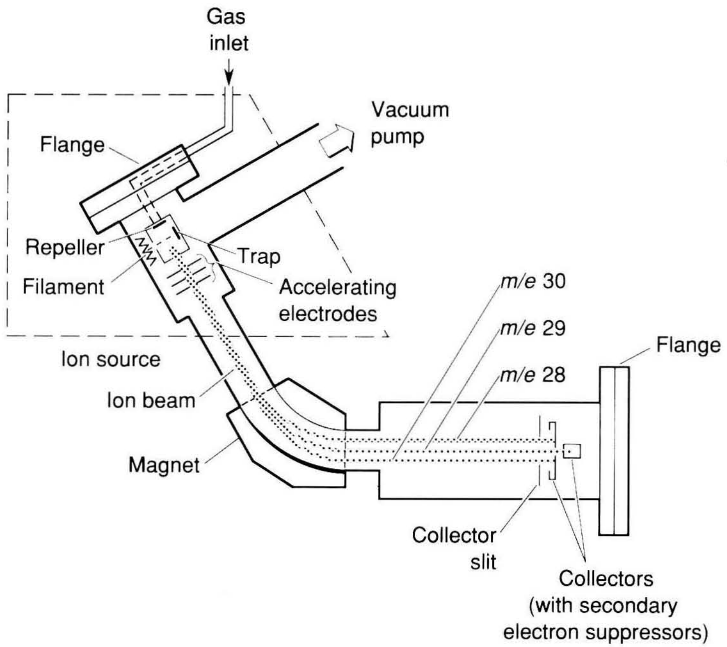

Figure 1 Schematic diagram illustrating operation of a double-collector mass spectrometer during isotope ratio analysis of N_2.

The positive ions are drawn out of the ion source by the combined effects of a small positive potential (e.g., 50 volts) on the repeller and a much larger negative potential across the accelerating electrodes (typically 3000–5000 volts). After passing through an exit slit, the ions enter the magnetic sector, consisting of the curved portion of the analyzer tube positioned between the poles of a permanent magnet or an electromagnet. Their path is curved due to the influence of the magnetic field, the radius of curvature depending on the mass and charge of the ions and their velocity (determined by the accelerating voltage) as well as the field strength. The two latter factors are held constant, so the ions are separated into three beams according to their mass-to-charge (*m/e*) ratios, the radius of curvature decreasing in the order $^{30}N_2^+$ (*m/e* 30) > $^{29}N_2^+$ (*m/e* 29) > $^{28}N_2^+$ (*m/e* 28). Additional beams of lower intensity are also formed (see Table I), due in part to the presence of O_2 and Ar in the N_2 under analysis and to residual gases in the mass spectrometer, including H_2O and CO_2.

Table I
Principal Ions Normally Present during Isotope Ratio Analysis of N_2

m/e	Ion
14	$(^{14}N^{14}N)^{2+}$
14.5	$(^{14}N^{15}N)^{2+}$
15	$(^{15}N^{15}N)^{2+}$
18	$(^{1}H^{1}H^{16}O)^{+}$
28	$(^{14}N^{14}N)^{+}$
29	$(^{14}N^{15}N)^{+}$
30	$(^{15}N^{15}N)^{+}$
32	$(^{16}O^{16}O)^{+}$
40	$(^{40}Ar)^{+}$
44	$(^{12}C^{16}O^{16}O)^{+}$

The intensities of the ion beams corresponding to m/e 28, 29, and 30 are directly related to the isotopic composition of the N_2 under analysis, and collection is accomplished using one or more insulated electrodes (anodes) with entrance slits and secondary electron suppression (to reduce noise). With the double-collector arrangement illustrated in Fig. 1, the ion beams corresponding to m/e 28 and 30 impinge on one collector (the multicollector), while the beam corresponding to m/e 29 impinges on the other (a Faraday cup positioned behind a slit in the multicollector). Focusing of the ion beams is accomplished by regulating the accelerating voltage (for instruments equipped with a permanent magnet) or the magnetic field strength (for instruments equipped with an electromagnet), which changes the m/e collected according to the equation

$$m/e = 4.82 \times 10^3 H^2 r^2 / P \qquad (1)$$

where H is the field strength in tesla, r is the radius of curvature in centimeters, and P is the accelerating potential in volts.

The current ($I = 10^{-9}$–10^{-12} A) generated by ions striking a collector is fed to an electrometer with a very high input resistance ($R = 10^{10}$–10^{12} Ω), which produces a proportional voltage ($V = IR$). The voltage is measured using a strip-chart recorder, a digital voltmeter, an integrating ratiometer, or a computer equipped with an analog-to-digital, or A/D, converter.

B. Parameters of Performance

The performance of a mass spectrometer can be measured in several ways. For an isotope ratio instrument, the primary parameters of interest are resolution, precision, accuracy, and sensitivity.

In mass spectrometry, resolution is defined as the ability to separate ion beams that differ in m/e ratio. It is most commonly calculated as $m/\Delta m$, where m is the nominal mass (actually m/e) for a particular peak in the mass spectrum, and Δm is peak width at 10 or 50% of the peak height. The resolution attainable with most isotope ratio mass spectrometers is <500, which is quite low compared to the resolution afforded by instruments used for exact mass measurements but is more than adequate for isotope analysis of N_2 and other gases of low molecular weight. Some increase in resolution can be achieved by reducing slit widths, but at the expense of sensitivity.

Precision refers to the reproducibility of an isotope abundance measurement. It is commonly expressed in terms of the coefficient of variation (relative standard deviation) for a series of measurements on a single sample (internal precision) or as the minimum detectable difference in isotope ratio between a pair of samples introduced sequentially from a dual-inlet system. Values quoted for commercially available isotope ratio mass spectrometers range from 0.001 to 0.00001 atom% ^{15}N. However, actual precision may be somewhat lower than the quoted value, particularly in analysis of small samples (i.e., those for which the inlet pressure is less than optimal) or samples highly enriched in ^{15}N.

The accuracy of a mass spectrometer for isotope abundance measurements is more difficult to evaluate than precision. Most commonly, evaluation is based on analyses of interlaboratory standards. Of course, the results obtained can be influenced by several factors besides the accuracy of the mass spectrometer, including sample size and purity and, particularly, faulty sample preparation technique, which can lead to isotope fractionation. A loss of accuracy often occurs at high enrichments, due to nonlinearity in the amplification of ion currents by the electrometer(s).

The sensitivity of a mass spectrometer can be defined as the minimum sample size required for optimal accuracy and precision. Conventionally, 0.5–5 mg N are required for N isotope analysis, but recent advances in automation (see Section II.F) have led to a dramatic gain in sensitivity, allowing routine analyses of <50 μg N. Among the factors that contribute to sensitivity are a decrease in the volume of the inlet system (e.g., through use of a smaller sample container) or in the speed of pumping of the ion source and an increase in (1) the size of the leak, (2) the efficiency of ionization, (3) the ion accelerating potential, or (4) the slit widths for source and collector(s).

C. Vacuum Systems

A fundamental requirement of any mass spectrometer is a vacuum system capable of maintaining very low pressure in the analyzer, typically <100 μPa in the magnetic sector and collector region. At such a pressure, the mean free path of ions formed in the source is sufficiently long to preclude collisions with gas molecules that would cause scattering and loss of energy and lead to ion–molecule reactions.

The vacuum system of a mass spectrometer must be designed to ensure rapid removal of un-ionized gas molecules from the analyzer, so pumping is carried out through a high conductance tube connected to the ion source housing. The speed of pumping should be sufficient to minimize memory from a previous sample, but not so high as to seriously limit sensitivity. In some cases, differential pumping is utilized to increase resolution, using a second pump connected to the collector housing.

The low pressures required for operation of a mass spectrometer cannot be attained with a mechanical (rotary) pump. A high vacuum pump must be used. The most common types are the diffusion pump, the ion pump, and the turbomolecular pump.

In the diffusion pump, a fluid having low volatility at room temperature (e.g., polyphenyl ether) is brought to boiling by a heater at the base of the pump. The vapor rises through the chimney assembly and is discharged from several ring-shaped nozzles to form a series of low-pressure vapor jets directed toward the base of the pump. Gas molecules at the inlet to the pump become entrained in the top jet and are carried downward, subsequently becoming entrained in lower jets. This causes the gas to be compressed to a pressure at which it can be removed by a mechanical (backing) pump. The vapor molecules in the diffusion pump condense on the water-cooled walls and return to the boiler.

The major limitation of the diffusion pump for mass spectrometry is backstreaming of the pump fluid, which can affect the background spectrum. To reduce this problem, a baffle or a trap containing liquid N_2 is attached to the inlet of the pump. Alternatively, an ion pump may be used, in which case there are no fluids of any kind. Rather, pumping is accomplished by applying a highly positive potential (3000–7000 volts) to a cylindrical anode placed between two flat cathode plates, usually made of Ti. Gas molecules that enter the pump are ionized by electrons from the anode; the ions impinge upon the cathodes with sufficient energy to be implanted, which causes sputtering of Ti. The sputtered Ti is deposited on a cathode or anode surface, where it acts as a getter film and adsorbs reactive gases such as N_2, O_2, and CO_2. To increase the path of the electrons, and hence the efficiency of ionization, the pump body is mounted between the poles of a permanent magnet. Ion pumps offer considerable convenience, because they operate without cooling water, a backing pump, or liquid N_2, and the flow of current indicates pressure, eliminating the need for a separate pressure gauge. However, ion pumps do not provide the strong pumping of a diffusion or turbomolecular pump, particularly in the case of the noble gases, which can cause serious instability (pressure bursts) with some designs.

Increasingly, the turbomolecular pump is being utilized in mass spectrometry. Pumping is accomplished through momentum transfer from the blades of a high-speed (15,000–100,000 rpm) rotor (turbine) arranged in series with a fixed stator, which compresses the gas molecules for removal by a mechanical backing pump. Compared to the diffusion pump, the turbomolecular pump has the advantage of producing a hydrocarbon-free vacuum without the need for a baffle or liquid N_2, and some models can be mounted in any orientation and operated without cooling water. The major limitations are a higher initial cost and the need for periodic bearing replacement, normally after 20,000–30,000 hr of operation.

D. Inlet Systems

The function of the inlet system is to present a steady stream of sample molecules to the ion source at an extremely low flow rate. Typically, this is accomplished using a vacuum manifold with a variable volume reservoir (a Toepler pump or metal bellows) and pressure gauge for pressure adjustment, connected to the ion source via a critical restriction (leak) to regulate the flow rate.

An important consideration in the design of the inlet system is whether gas flow into the ion source is to be molecular or viscous, which determines the necessary inlet pressure and the type of leak to be used. Molecular inlets find many applications in mass spectrometry, but viscous inlets are generally preferred in measuring isotope ratios, owing to the fact that isotope fractionation occurs during molecular flow. Viscous flow requires an orifice that is large relative to the mean free path of the gas molecules, so the volume of the sample reservoir is minimized to obtain an inlet pressure >1 kPa, and the leak is made by crimping a capillary tube, which provides a larger orifice than the leaks used in molecular inlets.

For isotope analyses requiring the highest accuracy and precision, such as those made to detect variations at the natural abundance level (Chapter 4), ratio difference measurements are made using a double- or triple-collector mass spectrometer equipped with a dual-inlet system. Figure 2 shows a schematic diagram of such a system. Among the components are metal bellows for equilibrating the pressures of sample and reference gases, matched viscous leaks, and a set of four changeover valves to sequentially admit sample and reference gases to the mass spectrometer and to a waste pump. During analyses, the changeover valves are actuated automatically at intervals preset by the operator for multiple comparisons of reference and sample under identical conditions.

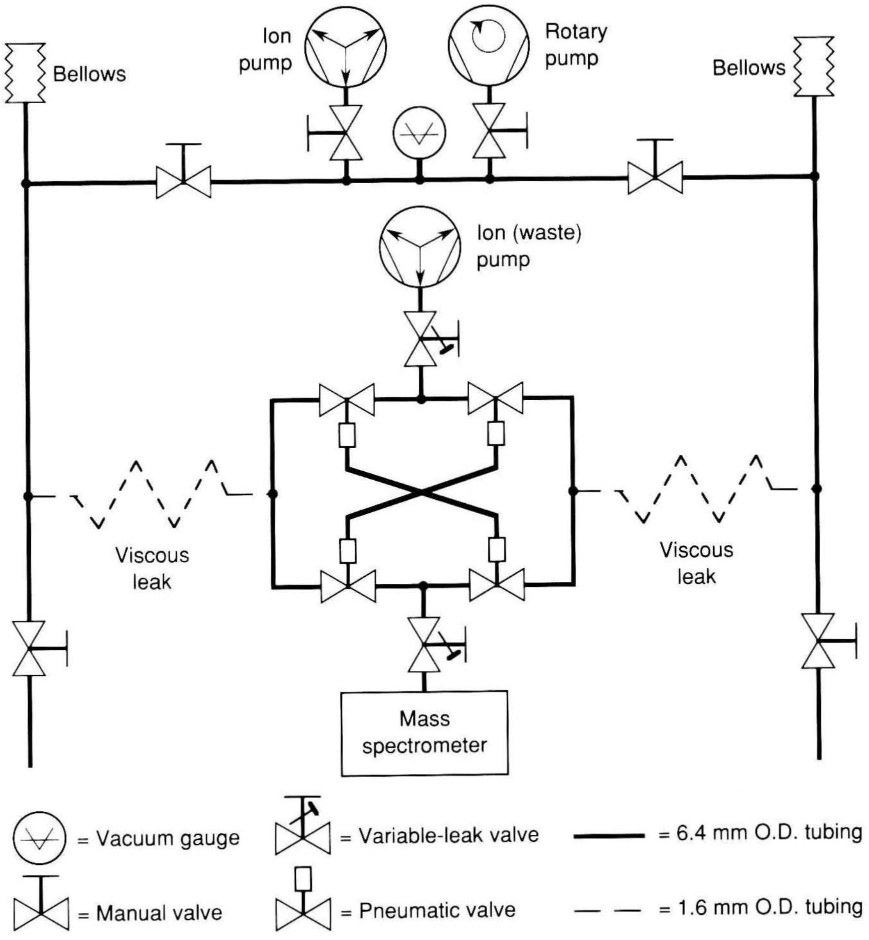

Figure 2 Schematic diagram of a dual-inlet system with matched viscous leaks.

E. Collector Systems

In addition to the double-collector system illustrated by Fig. 1, isotope analyses of N_2 can be performed using a single collector, in which case the ion beams of interest (normally *m/e* 28 and 29) are measured sequentially by regulation of the ion-accelerating potential or the current supplied to the electromagnet (for instruments so equipped). Single-collector measurements are generally considered to be less precise (by about an order of magnitude) than those made using double collectors because of fluctuations during analysis in sample pressure and composition and in the production of ions.

As originally proposed (Nier *et al.*, 1947), double-collector measurements were made by a null balance technique, in which the potentials from the two collectors are balanced against one another using a potentiometer controlled via decade voltage divider switches. This technique has largely been replaced by direct ratio measurements using an integrating digital ratiometer. The ratiometer, introduced in the early 1970s, has proven to be an outstanding advance in isotope ratio mass spectrometry. Not only is a digital display faster and more convenient than the null balance technique, but integration can markedly increase precision, and the output from the ratiometer can be utilized for on-line processing of data by a programmable calculator or computer.

An increasingly common feature of commercially available isotope ratio mass spectrometers is a triple-collector system. Originally developed for C and O isotopic analyses of CO_2 (Morales *et al.*, 1970), a triple collector allows simultaneous measurement of two different ratios, whereas sequential measurements would be required with a double-collector system. The additional capability is considerably less useful for analysis of N_2 than CO_2, because N isotopic abundance can usually be determined from a single ratio, and the determination can be made with equal precision using a double-collector system. However, there is sometimes a need for analysis of two different ratios to determine the relative proportions of all three isotopic species of N_2, as in measuring emission of N_2 into air during denitrification of ^{15}N-labeled NO_3^- (see Chapter 7). In such cases, the necessary ratio measurements can be made more rapidly with a triple-collector mass spectrometer than with a double-collector instrument.

F. Automation

As noted in the Introduction, the practice of mass spectrometry has undergone considerable change during the past two decades, due largely to utilization of solid-state electronics. Of particular significance has been the introduction of low-cost microcomputers, which are now commonly used for controlling the mass spectrometer and monitoring performance as well as for data acquisition. With some instruments, the inlet manifold may be operated under computer control so that unattended analyses can be performed on a series of gaseous samples (typically 20–50 samples per loading); however, in such cases, the preparation of samples (e.g., by hypobromite oxidation of NH_4^+ to N_2) is carried out manually and is off-line, which increases the risk of contamination by atmospheric N_2.

Conventionally, N isotope analyses are performed by the Rittenberg technique, which involves hypobromite oxidation of NH_4^+ to N_2 in the absence of air (see Section III.C). In 1978, a project was initiated by McInteer and Montoya at the Los Alamos National Laboratory to automate Rittenberg analyses of NH_4^+ salt samples. By 1980, they had developed a working prototype of an automated Rittenberg apparatus (ARA) allowing completely automated N isotope ratio analyses by mass spectrometry (McInteer and Montoya, 1981). With this system, NH_4^+ samples were placed in miniature plastic vials, 137 vials per sample tray. The tray was moved with a modified *x–y* plotter to sequentially position each vial beneath a pneumatically actuated reaction head designed to make a gas-tight seal with the vial. Air was removed by purging with Freon, hypobromite was added, and the N_2 liberated was allowed to flow through a liquid N_2 trap for removal of Freon before introduction to a manifold connecting a set of custom-fabricated micropneumatic valves. Pressure in the manifold was measured by a pressure transducer, and, if excessive, a valve to vacuum was opened momentarily. The N_2 was then admitted to the mass spectrometer (a double-collector instrument) for isotope ratio analysis. The entire process was under the control of a programmable calculator.

The automated system developed by McInteer and Montoya (1981) had capabilities far beyond those of any conventional isotope ratio mass spectrometer for analysis of ^{15}N; not only could it operate completely unattended, but analyses could be performed with only 25 μg N, at a rate of up to several hundred samples per day. Further improvements were made in the system, including the use of disposable plastic trays to contain samples and the capability for multiple loading of trays (McInteer *et al.*, 1984), and a private business was established to perform automated ^{15}N analyses for the scientific community (Isotope Services, Los Alamos, New Mexico). A commercial version of the ARA designed by McInteer and Montoya (1981), incorporating several refinements, has recently been developed (Mulvaney *et al.*, 1990; Mulvaney and Liu, 1991).

A simpler approach to automation of N isotope analysis involves interfacing an automatic N/C analyzer (ANCA) to a mass spectrometer (Otsuki *et al.*, 1983; Preston and Owens, 1983; Barrie and Workman, 1984; Marshall and Whiteway, 1985; Preston and McMillan, 1988; Barrie and Lemley, 1989; Barrie *et al.*, 1989; Egsgaard *et al.*, 1989; Harris and Paul, 1989; Schepers *et al.*, 1989; Barrie, 1991; Craswell and Eskew, 1991; Jensen, 1991). Operation of such a system (commonly referred to as ANCA-MS) is illustrated schematically in Fig. 3. Samples (normally in the form of a finely ground solid containing 20–150 μg N) are sealed into miniature Sn capsules for loading into the ANCA autosampler (typically a 50- or 66-place unit). From the autosampler, a capsule drops into a combustion chamber (a quartz tube heated to 1020°C through which a He carrier stream flows) containing a catalyst (e.g., Cr_2O_3 granules), finely divided CuO wire (to oxidize hydrocarbons), and Ag wool (to remove S and halogens). Concurrently, a pulse of O_2 is admitted to promote flash combustion of the Sn, which increases the temperature to around 1700°C, ensuring complete oxidation of the sample. The combustion products (CO_2, N_2, NO_x, and H_2O) are swept into a tube containing Cu wire at 600°C, where NO_x species are reduced to N_2, followed by $Mg(ClO_4)_2$ and Carbosorb® traps for removal of H_2O and CO_2. The N_2 is purified by gas chromatography, and a small fraction (≃1%) of the effluent is admitted to the mass spectrometer via capillary tubing connected to a three-way control valve for measurement of *m/e* 28, 29, and 30, from which both total N and ^{15}N may be determined (alternatively, total N may be determined from the ANCA if equipped with a thermal conductivity detector). The bulk of the effluent is exhausted through a coil of capillary tubing to avoid back diffusion of atmospheric N_2.

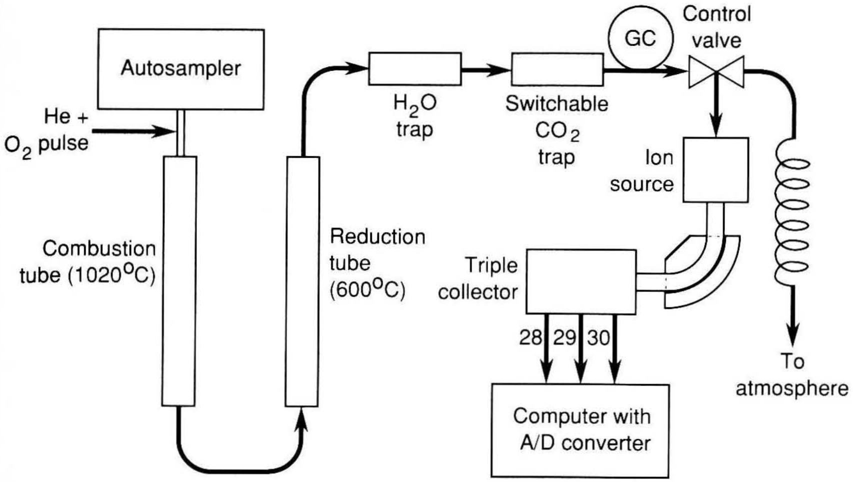

Figure 3 Schematic diagram illustrating ^{15}N analysis by ANCA-MS. (From Europa Scientific Ltd., Cheshire, United Kingdom.)

Use of ANCA-MS for N isotope analysis has expanded rapidly in recent years, and this trend will undoubtedly continue, due in part to the availability of commercial systems consisting of an ANCA, a dedicated magnetic sector mass spectrometer, and a microcomputer. These systems are simpler to operate and maintain than a conventional isotope ratio instrument and are remarkably compact, which makes them portable. They have been successfully used for routine analyses of plant and soil samples and have special value in marine and aquatic biology because analyses can be performed on submicrogram quantities of N (Owens and Rees, 1989), and operation is even possible aboard a ship at sea (Owens, 1988).

III. THE KJELDAHL–RITTENBERG TECHNIQUE FOR N ISOTOPE ANALYSIS

A. General Principles

For N isotope analyses by mass spectrometry, all forms of N in the sample under analysis must be converted into a suitable gas. Dinitrogen is preferred because of its low molecular weight and simple structure (in terms of both molecular and isotopic composition), and because it can be readily generated from a variety of organic and inorganic compounds, is chemically inert, and can be readily pumped from the mass spectrometer.

Although other methods have been used for N isotope analyses of solid or liquid samples by mass spectrometry (Bremner, 1965d; Fiedler and Proksch, 1975; Hauck, 1982; Fiedler, 1984), a three-step procedure is usually employed that involves (1) conversion of labeled N to NH_4^+-N, (2) oxidation of NH_4^+-N to N_2 by alkaline hypobromite in the absence of air, and (3) isotopic analysis of the N_2. This procedure was originally developed by Rittenberg and his colleagues (Rittenberg *et al.*, 1939; Rittenberg, 1948; Sprinson and Rittenberg, 1948, 1949) for use in medical research, but it has been applied more extensively in the agricultural and biological sciences.

Numerous modifications in analytical methodology have been described to improve the speed and convenience of N isotope analysis. Nevertheless, the three-step procedure for this analysis remains a complicated and time-consuming process, and considerable care is required at each step to avoid errors that can arise [for a thorough discussion of the possible errors, see Hauck (1982)]. Moreover, a substantial amount of N is needed (typically 1 mg), which complicates analyses in cases where sample size is limited or the material under study has a low N content (e.g., natural waters), or where the determination must be made on a specific form of N.

B. Conversion of Labeled N to NH_4^+-N

1. Principles

a. Total N Several methods are available for converting labeled N to NH_4^+. The method of choice is determined by the forms of N in the sample under study and by whether or not there is a need to distinguish between them. In most cases, conversion is carried out using the Kjeldahl method or one of its modifications.

The Kjeldahl method is a two-step procedure for determination of total N that involves (1) digestion with concentrated H_2SO_4 to convert organic forms of N to NH_4^+-N and (2) determination of the amount of NH_4^+-N in the digest. To increase the speed and completeness of digestion, additional substances are usually added to the H_2SO_4, most commonly a salt (normally K_2SO_4) to increase the temperature of digestion and a catalyst (Hg, Cu, or Se) to promote oxidation of organic matter. Quantitative determination of NH_4^+-N in the digest is usually accomplished by titration with standard acid of the NH_3 liberated during steam distillation of the digest with alkali.

There are numerous versions of the Kjeldahl method that involve various periods of digestion with different concentrations of salt and different catalysts (see Bremner and Mulvaney, 1982). Sample size also varies. Both macro- and semimicroversions of the Kjeldahl procedure have been employed for conversion of organic N to NH_4^+ for N isotope analysis. Macromethods involve digestion of a sample containing about 10 mg N in a 350- to 800-ml Kjeldahl digestion flask; semimicromethods involve digestion of approximately 1 mg N in a 30- to 100-ml flask. Semimicromethods are generally preferred for the preparation of samples for N isotope analysis, because they provide an appropriate amount of N for the analysis, and there is much less potential for cross-contamination of samples during distillation with a micro-Kjeldahl distillation apparatus than with a macrounit (Hauck, 1982).

During Kjeldahl digestion, H_2SO_4 is consumed in oxidation of organic C to CO_2, which leads to an increase in the salt concentration of the digest and the temperature of digestion. Loss of N occurs at temperatures above about 400°C, which requires a K_2SO_4 concentration of at least 1.3 g ml^{-1} of H_2SO_4 (Bremner and Mulvaney, 1982). This concentration can be attained under some conditions, particularly with Kjeldahl procedures that use high concentrations of K_2SO_4 (>1 g ml^{-1} H_2SO_4), and it is important to estimate the consumption of H_2SO_4 with such procedures so that sufficient acid can be added to compensate for the loss (Fiedler, 1984). This is seldom necessary with the concentrations of K_2SO_4 usually employed in Kjeldahl procedures for total N analysis of soils and plant materials (0.22–0.33 g K_2SO_4 ml^{-1} H_2SO_4), including the procedure described in Section III.B.3.a.

In ^{15}N tracer studies involving Kjeldahl digestion for determination of total ^{15}N, it is essential that all forms of N in the sample under study be quantitatively converted to NH_4^+-N. In some cases, a digestion period of 12–18 hr has been recommended to ensure the absence of volatile amines that interfere in isotope ratio analysis of N_2; however, the period of digestion required for complete conversion of organic N to NH_4^+ depends on the K_2SO_4 concentration, and no interference due to amines has been observed with Kjeldahl procedures for total N analysis of soils and plant materials involving digestion for 5 hr (Bremner, 1965d). In most investigations involving use of ^{15}N-labeled compounds, complete recovery of NO_3^- and NO_2^- is essential, because these forms of N will often have a substantially higher ^{15}N content than the organic N, and failure to recover them completely can lead to serious error in the isotope ratio analysis, even if their concentrations are low.

The two-step Kjeldahl method does not quantitatively recover compounds with N-N or N-O linkages, but several modifications have been described for inclusion of NO_3^- and NO_2^-, which contain N-O linkages (Bremner and Mulvaney, 1982). Of these, the permanganate-reduced Fe modification is generally preferred for use in studies involving N isotope analysis. In this modification, conversion of NO_3^--N and NO_2^--N to NH_4^+-N is carried out, before Kjeldahl digestion, by treatment of the sample with $KMnO_4$ to oxidize NO_2^- to NO_3^-, and then with H_2SO_4 and reduced Fe to generate H_2 for reduction of NO_3^- to NH_4^+.

b. Specific N forms Plants, soils, and natural waters contain various forms of inorganic and organic N, and the N isotopic composition of one or more specific N forms is often of interest in studies utilizing N isotope techniques. Several procedures have been developed to convert specific inorganic and organic forms of N in soil extracts and hydrolyzates to NH_4^+ for N isotope analysis, including exchangeable and nonexchangeable NH_4^+, NO_2^-, NO_3^-, α-amino acids, amino sugars, hydroxyamino acids (serine and threonine), and urea. Some of these procedures are discussed in Chapters 4 (Section II.A.3) and 7 (Section II.B); additional information can be found in publications by Bremner (1965b,c) and Keeney and Bremner (1967). It suffices here to note that all of the procedures involve steam distillation of NH_4^+ and that most have application in analysis of plant materials and natural waters.

c. Distillation and collection of NH_4^+-N Following conversion of labeled N to NH_4^+, the NH_4^+ must be separated from the reagents used for the conversion and from other constituents of the sample to eliminate any substance that would decompose the hypobromite used to oxidize NH_4^+ to N_2 or effect contamination of the N_2 generated by this oxidation. In most Kjeldahl procedures, separation is carried out by steam-distilling the digest with concentrated NaOH; the NH_3 liberated is collected in H_3BO_3-indicator solution for titrimetric determination of NH_4^+-N. Distillation is usually performed using an apparatus fabricated largely, if not entirely, from borosilicate glass. This can lead to cross-contamination of samples, because a small amount of NH_3-N adheres to the glass whenever a sample is distilled, and some of this adhering N will be displaced during distillation of the following sample. If the two samples differ in their content of ^{15}N, the second sample will become "cross-contaminated." The extent of contamination will depend on the amount of N distilled, its ^{15}N content compared to that of the previous sample, and the internal surface area of the distillation apparatus used. Several procedures have been employed to minimize cross-contamination during steam distillation of NH_4^+ for N isotope analysis (Mulvaney, 1986), and some workers have utilized stainless-steel components for the distillation apparatus (Buresh *et al.*, 1982; Pruden *et al.*, 1985; Vanden Heuvel and Giamalva, 1988).

d. Concentration of NH_4^+-N With semimicro-Kjeldahl procedures, the volume of distillate after titration typically exceeds 40 ml, which must be reduced to 2–5 ml for transfer to a glass vial, and subsequently evaporated to dryness, for hypobromite oxidation of NH_4^+ to N_2 by the procedure described in Section III.C.3. Evaporation is easily accomplished in open beakers on a steam plate or hot plate, but acidulation of the titrated distillate is essential to prevent loss of NH_4^+-N upon heating, and this can lead to contamination by atmospheric NH_3, which is present throughout buildings with central heating and air conditioning owing to the use of NH_3-based cleaning solutions and floor waxes (Edwards, 1978). Various precautions can be taken to minimize exposure to ambient NH_3 during evaporation of distillates (see Section III.B.4.c). The most effective approach is to avoid such exposure altogether, either by use of a specially designed chamber supplied with NH_3-free air (Hauck, 1982) or by performing evaporations under vacuum (Kelley, 1989).

2. Materials Required

a. Special apparati

1. Aluminum block digester or micro-Kjeldahl digestion stand.
2. Pyrex® digestion tubes (50–250 ml) and/or Kjeldahl flasks (100 ml, with Ŧ 24/40 inner joint).
3. Semimicrodistillation apparatus (Fig. 4). The unit illustrated in Fig. 4 is a modified version of the apparatus described by Bremner (1965b) for determination of inorganic forms of N in soils and soil extracts and is primarily designed for the same purpose (Mulvaney, 1986). An adapter allows attachment of a 100-ml Kjeldahl flask equipped with a ground-glass joint (the joint should not be greased), which serves as the distillation chamber. Steam for distillation is generated by boiling distilled, deionized (DI) water in a 5-liter flask containing a few Teflon boiling chips and equipped with an electric heating mantle controlled by a variable transformer. Before use, the apparatus should be steamed out for about 10 min, during which the transformer is adjusted so that distillate is collected at the rate of 7–8 ml min^{-1}, at a temperature no higher than 22°C. A small plastic cap is installed on the outlet end of the condenser to prevent conden-

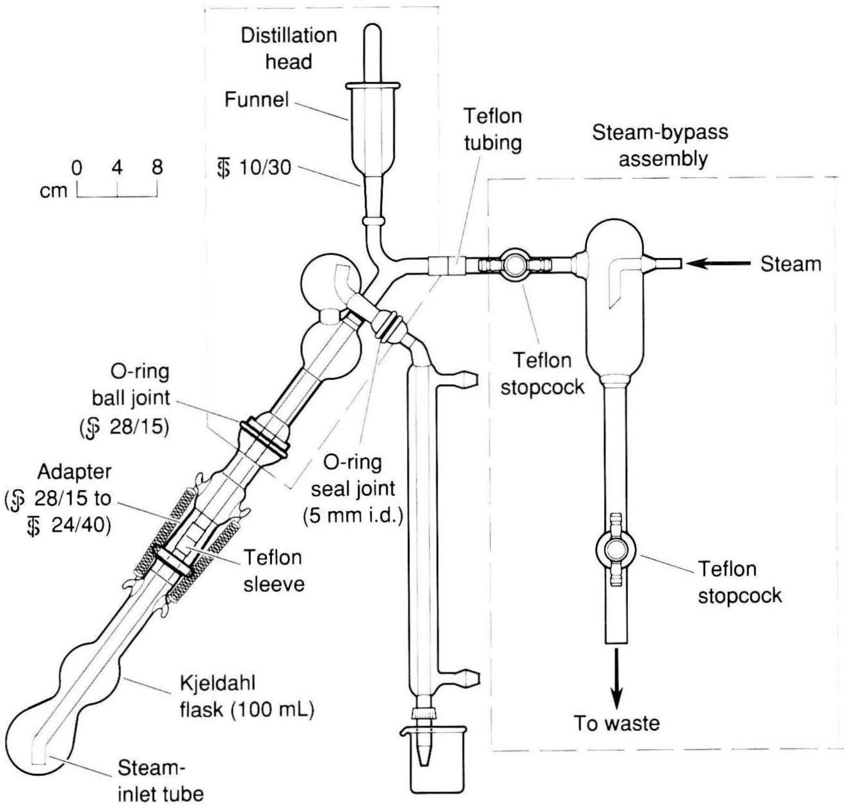

Figure 4 Steam distillation apparatus.

sate that collects on the outside surface of the condenser from contaminating the distillate.

4. Microburette (5 ml, graduated at 0.01-ml intervals) or automatic titrator.
5. Hot plate or other apparatus for concentration of distillate.
6. Shell vials (17 mm OD, 60 mm long). Kimble No. 60930L-2 is satisfactory.

b. Reagents[1]

1. Sulfuric acid, concentrated (18 *M*).
2. Potassium permanganate solution. Dissolve 25 g $KMnO_4$ in 500 ml DI water. Store in an amber bottle.
3. Sulfuric acid (9 *M*). To 500 ml DI water in a 2-liter Pyrex® flask, slowly add 500 ml concentrated H_2SO_4. Mix carefully and cool.

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[1] After Bremner and Mulvaney (1982).

4. Reduced Fe powder. The certified reagent available from Fisher Scientific is acceptable. Before use, it should be sieved to remove material coarser than 150 μm.
5. Potassium sulfate–catalyst mixture. Powder 20 g $CuSO_4 \cdot 5H_2O$ by grinding in a mortar, and mix intimately with 2 g Se and 200 g K_2SO_4 (powder).
6. Sodium hydroxide solution, approximately 10 *M*. To 12 liters DI water in a heavy-walled 20-liter Pyrex® bottle, add 6.0 kg NaOH pellets. Dissolve the pellets with vigorous stirring from a motorized stirrer. Cool the solution, bring the volume to 15 liters by adding DI water, and mix thoroughly with the motorized stirrer. Stopper the bottle during storage.
7. Boric acid-indicator solution. Add 400 g H_3BO_3 to 18 liters DI water in a 20-liter Pyrex® bottle marked to indicate a volume of 20 liters, and stir vigorously with a motorized stirrer to dissolve the H_3BO_3. Then add 400 ml indicator solution (prepared by dissolution of 0.495 g bromocresol green and 0.33 g methyl red in 500 ml ethanol), and bring the volume to 20 liters with DI water. With continuous stirring, adjust the pH to approximately 5.0, or until the solution assumes a reddish purple tint, by cautiously adding 1 *M* NaOH (if excess NaOH is added, the pH can be reduced by adding dilute HCl). Dispense the solution through Teflon tubing.
8. Dilute H_2SO_4, 0.005 *M* standard (for titration) and 0.4 *M* (for acidulation of distillates).
9. Formic acid, 1 *M*. Dilute 10.5 ml concentrated (23.6 *M*) formic acid to 250 ml with DI water in a volumetric flask.
10. Ethanol, 16.4 *M* (95%).

3. Description of Procedures

a. Digestion[2] Place a sample of soil, plant material, or solution ($\leq$20 ml natural water or 0.5 *M* K_2SO_4 soil extract) containing about 1 mg N in a Kjeldahl flask or digestion tube, add 1 ml $KMnO_4$ solution (by dispenser or pipette), and swirl to mix completely. Allow the sample to stand for about 30 sec; then pipette 2 ml 9 *M* H_2SO_4 down the side of the flask or tube, taking care that no sample material is left on the wall. After 5 min, add 0.50 ± 0.01 g reduced Fe powder through a funnel with a stem that reaches to within 1–2 cm of the mixture at the bottom. Swirl the flask or tube to bring all of the Fe into contact with the acid, allow it to stand for about 15 min (preferably in a fume hood), and apply gentle heat ($\simeq$90°C) for 45 min using the digestion stand or block in a fume hood. Allow a few minutes for cooling; then add 1.5 g K_2SO_4–catalyst mixture (through a long-stemmed funnel) and 4 ml concentrated H_2SO_4, and heat at about 150°C until the water is removed and white fumes appear (30 min to 1 hr for soil or plant samples; up to 4 hr for solution samples). Increase the heat (to about 250°C) until the color of the mixture becomes yellowish green, and then boil the mixture gently (340–360°C) for 5 hr.

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[2] After Bremner and Mulvaney (1982).

b. Distillation[3] After digestion is complete, allow time for cooling, and then add about 10 ml DI water to the digest from a wash bottle. If digestion was performed using an Al block, mix the diluted digest using a vortex mixer, and transfer the mixture to a 100-ml Kjeldahl flask equipped with a Ŧ 24/40 inner joint (transfer is aided if the digest has not cooled below 100°C; transfer is unnecessary if the tubes are equipped with ground-glass joints for connection to the distillation apparatus). Repeat the transfer three times with a total of 15–20 ml water. Prepare the steam distillation apparatus for use by opening the lower stopcock on the steam-bypass assembly and closing the upper stopcock, which is connected to the distillation head (Fig. 4). Add 5 ml H_3BO_3-indicator solution to a 100- or 150-ml beaker marked to indicate a volume of 35 ml, and position the beaker under the condenser of the distillation apparatus so that the tip of the condenser is in contact with the side of the beaker and about 1 cm below the top. Then add 20 ml 10 M NaOH to the funnel affixed to the top of the distillation head, attach the Kjeldahl flask to the distillation apparatus, and lift the peg stopper in the funnel to allow the NaOH to flow slowly into the flask. When the funnel has been emptied, rinse it rapidly with about 5 ml DI water from a wash bottle, and allow this water to run into the flask. Then seal the funnel with the peg stopper, and immediately commence distillation by opening the upper stopcock on the steam-bypass assembly and closing the lower stopcock. When the volume of distillate reaches 35 ml, rinse the tip of the condenser, and stop the distillation by opening the lower stopcock on the steam-bypass assembly. Determine NH_4^+-N in the distillate by titration with 0.005 M H_2SO_4 (1 ml 0.005 M H_2SO_4 is equivalent to 140 μg NH_4^+-N). At the end-point, the color changes from green to a faint pink.

After removing the Kjeldahl flask from the distillation apparatus, attach a flask containing 1 ml 1 M formic acid, and carry out steam distillation for 1 min. Then detach the flask, and replace it with one containing 25 ml ethanol. Steam-distill the ethanol for 3 min.

c. Concentration of distillate Following titration, add 0.2 ml 0.4 M H_2SO_4 to the distillate, and evaporate to dryness at a temperature of 85–90°C.

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[3] After Bremner and Mulvaney (1982), Bremner (1965b), and Mulvaney (1986).

Dissolve the residue in 2–3 ml DI water, transfer the solution to a shell vial, and evaporate to dryness (85–90°C). Store the vials in a covered box.

4. Comments

a. Digestion Several types of micro-Kjeldahl digestion stands are available commercially, but some require modification to perform satisfactorily, and others are unsuitable because they provide too much or too little heat. According to Bremner and Mulvaney (1982), digestion stands for semimicro-Kjeldahl analyses should meet the following requirements: (1) the heaters should have individual controls; (2) at full heat, each heater should bring 25 ml of water to a rolling boil in about 2.5 min; (3) the Kjeldahl flask should be supported so that its neck is <45° from the horizontal; (4) heating of the flask should only occur from directly below the region occupied by liquid; (5) a Kjeldahl digest should boil steadily for several hours, with H_2SO_4 condensing about one-third of the way up the neck; and (6) some means should be provided to dispose of acid fumes from digestion, such as a glass fume duct manifold connected to an aspirator vacuum pump. During digestions, the Kjeldahl flasks should be swirled occasionally to ensure that any sample material deposited on the walls by spattering is returned to the digestion mixture.

Aluminum block digesters provide better temperature control than digestion stands and are more convenient, particularly when equipped with a programmable temperature controller. Units designed for 50- to 250-ml digestion tubes may be used. If equipped with ground-glass joints, 250-ml tubes are conveniently attached to the distillation apparatus for collection of NH_4^+-N in the digest. If necessary, fuming can be controlled by fitting an exhaust manifold to the tubes during digestion or by placing funnels in their necks.

A digestion period of 5 hr is recommended to ensure complete conversion of N to NH_4^+. A shorter period may lead to inaccurate N isotope analyses, particularly for ^{15}N-labeled soil (Hauck, 1982).

For highest precision in total N analysis of soil or plant materials, samples should be dried, ground to pass through a 150-μm screen, mixed thoroughly, and weighed to 0.1 mg with an analytical balance. However, digestions can also be performed on moist samples, and this is sometimes necessary to avoid loss of N, as can occur upon drying of soil that contains NH_4^+ and NO_2^- (Bremner, 1965b).

Serious loss of N occurs if the procedure described is performed on soil extracts obtained using 1 or 2 M KCl. No such difficulty has been observed in analysis of 0.5 M K_2SO_4 soil extracts.

Controls should be included with each set of digestions so that total N analyses can be corrected for N in the reagents used. Nevertheless, care is required to avoid inadvertent addition of N, which can cause serious error in isotope ratio analyses. The most likely source of such N is the Fe used in reduction of NO_3^- to NH_4^+; therefore, a product certified to have a low N content (<30 μg g^{-1}) is specified. Contamination can also arise from the presence of weighing paper during digestion (Hauck, 1982), which precludes the use of this material to aid the transfer of solid samples prior to digestion for N isotope analysis.

Recovery of organic N should be checked by performing digestions on accurately known amounts of *tris*(hydroxymethyl)aminomethane (tradename, THAM®), which is available from several commercial sources as a highly purified acidimetric and N standard. Recovery of NO_3^--N and NO_2^--N should be checked using reagent-grade KNO_3 and $NaNO_2$ of known purity; suitable products are available from J. T. Baker (Phillipsburg, New Jersey) under the tradename, "Baker-Analyzed"®. In all cases, recovery should be at least 98%.

b. Distillation Various types of steam distillation units have been employed for determination of NH_4^+ in Kjeldahl digests of soils and plant materials (Bremner and Mulvaney, 1982), but not all are suitable for the preparation of samples for N isotope analysis. To reduce cross-contamination, the use of rubber or plastic components is to be avoided, and the internal surface area should be kept to a minimum (Reeder *et al.*, 1980). The apparatus illustrated in Fig. 4 was designed so that it can be rapidly disassembled and washed between distillations (Bremner, 1965b), but this is unnecessary if formic acid and ethanol are distilled between samples as described, or if a preliminary distillation is performed using an aliquot of the digest or a duplicate digest (Pruden *et al.*, 1985; Mulvaney, 1986). In the latter case, the amount of N in the sample may be determined by titrating the first distillate, while the second distillate, to be retained for isotope ratio analysis, can be collected using dilute (e.g., 0.025 *M*) H_2SO_4 instead of H_3BO_3-indicator solution.

Because of the need to perform additional procedures to minimize cross-contamination, distillations of Kjeldahl digests are much more time-consuming when they are to be followed by N isotope analyses of NH_4^+ in the distillates. To save time, two distillation units can be operated simultaneously, which allows up to 40 distillates to be collected for N isotope analysis in a normal working day.

As specified, distillates are collected in H_3BO_3-indicator solution so that quantitative determinations can be made by titration with standard H_2SO_4 (Bremner, 1965d). Collections may be performed using either beakers or Erlenmeyer flasks. Beakers are preferred in cases involving use of an automatic titrator, or where concentration of distillates is to be carried out by open-air evaporation. To avoid cross-contamination, glassware for collecting distillates should be acid-washed before use (Vanden Heuvel and Giamalva, 1988), or collections should be performed using disposable polyethylene or polypropylene beakers.

Isotope fractionation can arise through incomplete distillation of NH_4^+-N or loss of N during collection (Hauck, 1982). Therefore, care must be taken to collect at least 30 ml of distillate and to regulate the rate of distillation so that the temperature of the distillate does not exceed 22°C.

The H_2SO_4 used for titration can be standardized using primary standard-grade THAM®. The THAM® can be weighed directly (after drying for 2 hr at 105°C), but it is usually more convenient to pipette aliquots from an aqueous solution. Such a solution is stable for several months if stored in a refrigerator, but the container should be stoppered tightly to exclude atmospheric CO_2, which is slowly absorbed.

c. Concentration of distillate Evaporation of distillates for N isotope analysis should be carried out rapidly and without cross-contamination, contamination by atmospheric NH_3, or loss of N (Hauck, 1982).

Open-air evaporation of distillates to dryness can be accomplished within 3–4 hr if they are collected in beakers as specified, or in a somewhat shorter period by directing a stream of dry, NH_3-free air onto the surface of each distillate from a forced-air system (Hauck, 1982). Forced-air evaporation is essential for concentration of distillates in Erlenmeyer flasks, and it speeds the final drying of samples in vials, particularly if the vials are contained in a heated Al block (Lober *et al.*, 1987).

To avoid cross-contamination, distillates must not be heated so strongly that they boil or spatter outside the container upon drying. If evaporation is performed using a forced-air system, the delivery tubes must be cleaned thoroughly after use (Hauck, 1982) or must be replaceable.

To minimize contamination by atmospheric NH_3, all materials that liberate NH_3 or amine vapors should be excluded from the laboratory (Fiedler and Proksch, 1975), and care should be taken to ensure that concentration of distillates is not carried out under conditions that promote exposure to ambient NH_3, such as will occur during open-air evaporation in a fume hood (Edwards, 1978). Contamination by atmospheric NH_3 may be reduced by forced-air evaporation; however, the airstream often contains trace amounts of NH_3, which must be removed completely with an acid scrubbing system (Lober *et al.*, 1987). Whatever technique is used to concentrate distillates, contamination by ambient NH_3 should be checked using ^{15}N-labeled $(NH_4)_2SO_4$.

Serious loss of N can occur if distillates are concentrated without acidulation, and even if sufficient N remains for isotope ratio analysis with the mass spectrometer, the ^{15}N content may be increased substantially by isotopic fractionation. For acidulation, H_2SO_4 is recommended rather than HCl, because loss of N occurs when NH_4Cl is heated above 90°C, whereas $(NH_4)_2SO_4$ is stable at temperatures up to 235°C (Hauck, 1982). The amount of H_2SO_4 used for acidulation should not exceed the addition specified, as there will be very little, if any, loss during evaporation, and excess acid causes decomposition of hypobromite used in oxidation of NH_4^+-N to N_2 for isotope ratio analysis (see Section III.C).

C. Conversion of NH_4^+-N to N_2

1. Principles

The usual approach for converting NH_4^+-N to N_2 for isotope ratio analysis by mass spectrometry utilizes the reaction between alkaline hypobromite and NH_4^+, which is carried out under vacuum to exclude atmospheric N_2. This reaction is typically represented as

$$2\ NH_3 + 3\ OBr^- \rightarrow 3\ Br^- + 3\ H_2O + N_2$$

however, conversion of NH_4^+-N to N_2 is not quantitative. In addition to N_2, small amounts of N_2O and NO_3^- are formed (see Bremner, 1965d; Hauck, 1982).

The reaction of hypobromite with NH_4^+ has been employed for N isotope analysis since the pioneering work of Rittenberg and his colleagues in the 1930s. With the classical technique developed by Sprinson and Rittenberg (1948, 1949), the reaction is carried out in a Y-shaped tube that can be tilted to bring the hypobromite solution into contact with a liquid sample (Fig. 5). With various modifications (e.g., Bremner, 1965d; Edwards, 1978; Hauck, 1982), this technique has been applied extensively in the agricultural and biological sciences. It is, however, a tedious and time-consuming procedure, owing to the need for complete degassing of liquid solutions before reaction and for cleaning and reuse of the Y-tubes, and considerable care is required to control effervescence during initial evacuation and avoid contamination from external air leaks. The latter problem has been reduced considerably through the use of vacuum stopcocks and fittings that utilize O-ring seals. Moreover, these seals have allowed the development of systems in which hypobromite oxidations are performed on dried NH_4^+-salt samples in disposable glass vials (Ross and Martin, 1970; Porter and O'Deen, 1977; Buresh *et al.*, 1982; Pruden *et al.*, 1985). Such systems offer greater speed and convenience than the classical Rittenberg technique using Y-tubes.

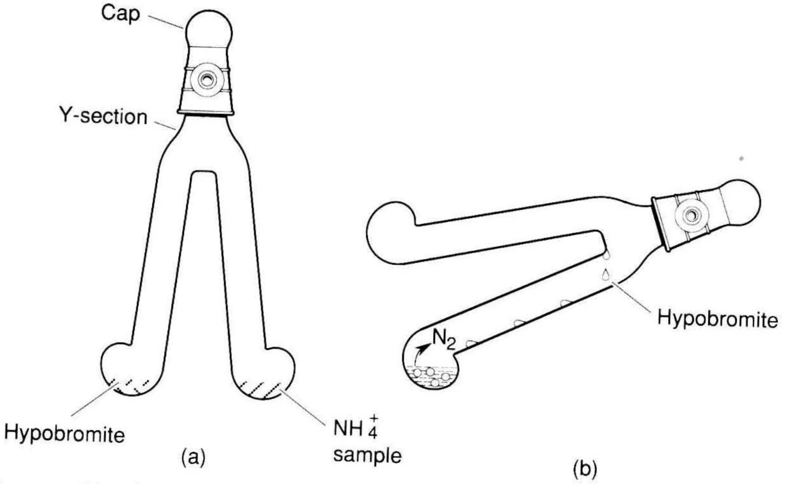

Figure 5 Rittenberg Y-tube. (a) Upright position for evacuation and degassing; (b) Tilted for hypobromite oxidation of NH_4^+-N to N_2.

Use of hypobromite to convert NH_4^+-N to N_2 for isotope ratio analysis is complicated by the fact that this material is unstable, decomposing to form bromide and O_2 ($2\ OBr^- \rightarrow 2\ Br^- + O_2$). For this reason, hypobromite cannot be purchased commercially but must be prepared by adding Br_2 to an aqueous solution of an alkali metal hydroxide. Sodium hydroxide has traditionally been used in the preparation of hypobromite solutions for N isotope analysis, but LiOBr was recommended by Ross and Martin (1970) for analysis of dried samples in glass vials, based on work by Polak *et al.* (1966) indicating that LiOBr is more stable than NaOBr. The LiOBr reagent recommended by Ross and Martin (1970) has been employed with modified versions of their technique (Porter and O'Deen, 1977; Buresh *et al.*, 1982; Pruden *et al.*, 1985), and the same reagent is used with the procedure described in Section III.C.3. However, there are reports that LiOBr is no more stable than NaOBr (Edwards, 1978; Hauck, 1982), which suggests that either reagent can be used to generate N_2 from dried NH_4^+-salts. Regardless of whether LiOBr or NaOBr is used, the reagent should be stored in a refrigerator to prevent thermal and photodecomposition (Polak *et al.*, 1966), and care should be taken to eliminate any possibility of contamination of samples by metals, which catalyze the decomposition of hypobromite (Polak *et al.*, 1966; Hauck, 1982). Care should also be taken to ensure that samples do not contain an excessive amount of acid, because this can lead to evolution of Br_2 upon treatment with hypobromite ($Br^- + OBr^- + 2\ H^+ \rightarrow H_2O + Br_2$).

The N_2 generated by hypobromite oxidation of NH_4^+ must be purified to remove hypobromite and water vapor, and this is accomplished by freezing the sample container (Y-tube or vial) in a low-temperature bath after reaction is complete and by passing the gas through a cold trap before admission to the mass spectrometer. Dry ice mixtures have been used for this purpose, but liquid N_2 is preferred because it freezes out N_2O, which interferes with ^{15}N analysis by undergoing partial decomposition in the ion source to form NO and N_2.

2. Materials Required

a. *Special apparati*[4]

1. Shell vial containing dried NH_4^+-salt sample (see Sections III.B.2.a and III.B.3.c).
2. Hypobromite conversion apparatus (Fig. 6a). The unit illustrated in Fig. 6a is designed to allow evacuation of a shell vial containing an NH_4^+-salt sample, introduction of LiOBr to convert the NH_4^+-N to N_2, and transfer of the N_2 formed to the inlet system of the mass spectrometer. Evacuation is accomplished using a dedicated pumping system rather than the inlet pumping system of the mass spectrometer so that conversion of NH_4^+-N to N_2 can be carried out while isotope ratio analysis is being performed on N_2 from a previous sample. Liquid N_2 traps are employed to exclude water and hypobromite vapors from the pumping system and purify sample N_2 before admission to the mass spectrometer. The LiOBr reservoir and the fitting to which it attaches (Fig. 6b) are noncommercial fabrications. Connection of components is accomplished using stainless-steel compression and O-ring fittings.

b. *Reagents*[5]

1. Alkaline LiOBr solution. Dissolve 40 g $LiOH \cdot H_2O$ in 400 ml DI water in a 500-ml Erlenmeyer flask using a magnetic stirrer. Then immerse the flask in a container of crushed ice, and allow the solution to cool to 5°C. With constant stirring, add 13.3 ml Br_2 dropwise over a period of 30 min. When the Br_2 has dissolved completely, remove the flask from the ice bath, and transfer the LiOBr solution to the reservoir bulb (Fig. 6b) by drawing it up through the delivery tube while evacuating the bulb via the top stopcock. When the transfer is complete, close the bottom stopcock, and clean the delivery tube with DI water from a rinse bottle (a variable-volume dispenser bottle with the stem tip removed and the measuring chamber shortened to serve as a collection cup). Then disconnect the bulb from vacuum, and purge dissolved air from the LiOBr solution by introducing He (gauge pressure = 10–20 kPa, flow rate = 0.5–1 liter min^{-1}) through the dispersion tube and allowing it to exhaust via the top stopcock. Continue purging for 1 hr; then close the top stopcock and, after a few seconds, close the stopcock connected to the dispersion tube. Before use, the bulb is positioned in the O-ring fitting on the conversion apparatus as illustrated in Fig. 6b, and the nut is finger-tightened to compress the O-ring against the outside of the delivery tube. If the bulb is stored in a refrigerator, the reagent will retain its activity for at least 6 mo. One milliliter of this solution will oxidize about 2 mg NH_4^+-N to N_2.
2. Liquid N_2.

3. Description of Procedure

With stopcock 2 and valve 2 (Fig. 6a) closed (valve 3 is also closed; stopcock 1 and valve 1 are open), insert a shell vial containing the NH_4^+-salt sample into the O-ring fitting on the conversion apparatus (Fig. 6b), and finger-tighten the nut. Open stopcock 2 to begin evacuation of the vial, and when vacuum gauge 1 indicates a pressure <25 Pa (<200 mtorr), position a Dewar flask containing liquid N_2 so that the vial is immersed to a depth of approximately 1 cm. When vacuum gauge 1 indicates a pressure <100 mPa (<1 mtorr), close stopcock 2, and briefly open the bottom stopcock on the LiOBr reservoir to deliver about 1 ml of reagent. Remove the Dewar flask from the sample vial, and warm the vial with water (≃25°C) in a beaker until all of the ice inside has melted. Then dry the outside of the vial by wiping it with a tissue, and again position the Dewar flask containing liquid N_2 to freeze the vial. When freezing is complete (within ≃30 sec, or as indicated by a crackling sound), close valve 1, and then open valve 2 to allow the flow of gas through cold trap 2. If vacuum gauge 2 indicates normal pressure (e.g., <1 kPa), open valve 3 to admit the gas to the inlet system of the mass spectrometer (if pressure is excessive, keep valve 3 closed, and proceed by closing valve 2 and opening valve 1). When transfer is complete (normally within 5–10 sec), close valves 2 and 3, and open valve 1 to restore vacuum. Remove the sample vial from the O-ring fitting and discard it. Before connecting another vial, clean the LiOBr delivery tube with DI water from the rinse bottle, and dry the inside and outside of the tube with a tissue (drying need not be complete).

4. Comments

The apparatus illustrated in Fig. 6a is a direct modification of the hypobromite conversion system described by Buresh *et al.* (1982). The major

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[4] After Porter and O'Deen (1977) and Buresh *et al.* (1982).

[5] After Ross and Martin (1970) and Buresh *et al.* (1982).

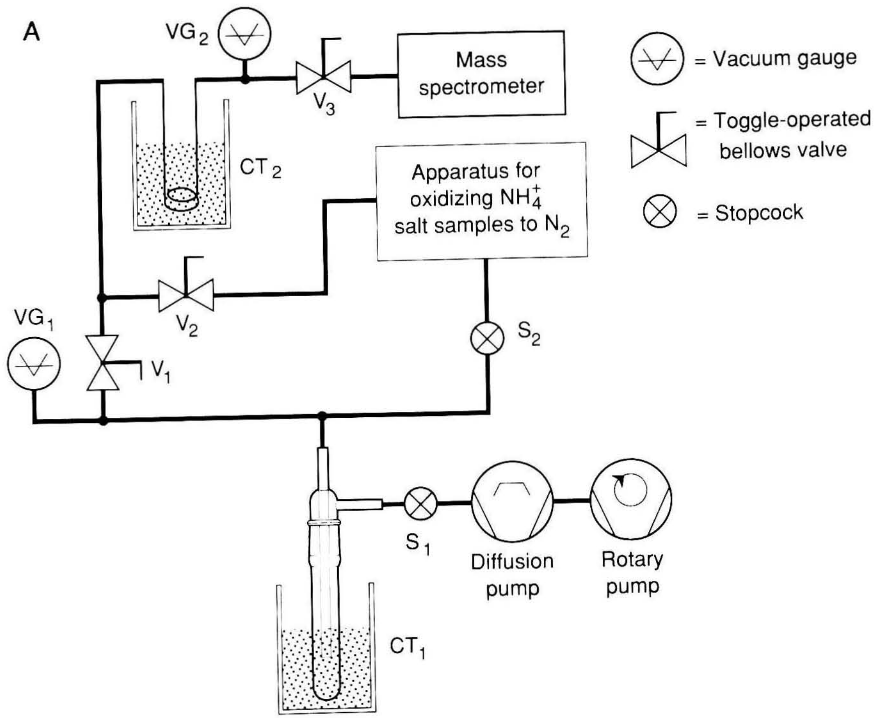

Figure 6 Hypobromite conversion apparatus. (A) Schematic diagram of complete apparatus. CT = cold trap (liquid N_2). CT_1 is a Pyrex® vacuum trap with ground-glass joint. CT_2 is a coiled U-trap (30 cm long, 3.2 mm OD stainless-steel tubing). VG = vacuum gauge. VG_1 is a thermocouple gauge. VG_2 is a low-volume pressure transducer or Hg manometer. S = stopcock (high vacuum, with Teflon plug and O-ring seals). S_1 has a 0–10-mm orifice. S_2 has a 0–5-mm orifice. (B) Diagrammatic representation of components used to convert NH_4^+-N to N_2. The O-ring fitting required can be fabricated from a Cajon Ultra-Torr® union (SS-10-UT-6; Cajon Company, 9760 Shepard Road, Macedonia, Ohio 44056) by (1) boring out one end to 17 mm; (2) cross-drilling the hex flat; (3) countersinking the drilled sides of the hex flat to accept two Ultra-Torr® adapters (one SS-4-UT-A-4 and one SS-6-UT-A-6); (4) silver-soldering the adapters to the union; and (5) welding a stainless-steel strap to the union for mounting.

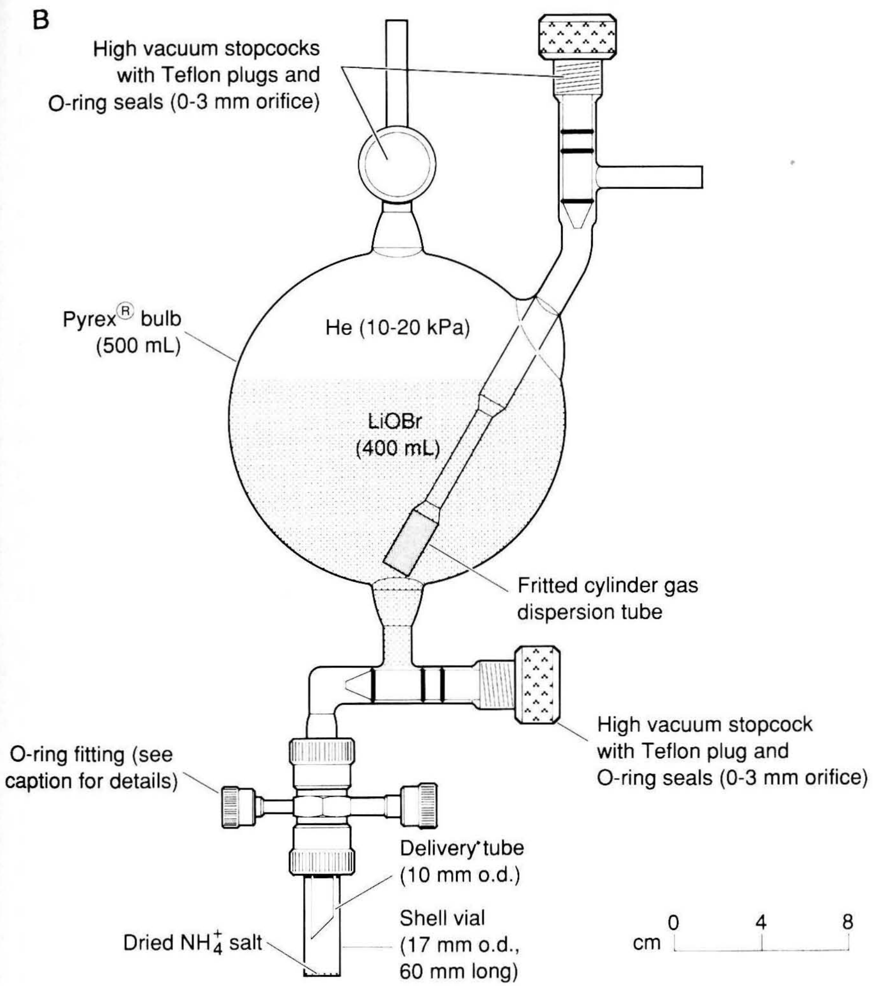
B
High vacuum stopcocks with Teflon plugs and O-ring seals (0-3 mm orifice)
Pyrex® bulb (500 mL)
He (10-20 kPa)
LiOBr (400 mL)
Fritted cylinder gas dispersion tube
High vacuum stopcock with Teflon plug and O-ring seals (0-3 mm orifice)
O-ring fitting (see caption for details)
Delivery tube (10 mm o.d.)
Shell vial (17 mm o.d., 60 mm long)
Dried NH4+ salt
cm
0
4
8

Figure 6 *(continued)*

modifications involve the addition of a port to the O-ring fitting for evacuation of sample vials via a glass stopcock, and the use of an oil diffusion pump as well as a rotary pump. The former modification was made to speed evacuation and reduce fouling of bellows valves by the dried residue from H_3BO_3-indicator solution. The latter modification was dictated by the need for a pumping system capable of rapid and repeated evacuation from atmospheric pressure to <100 mPa (<1 mtorr). Pressures of this magnitude can be achieved with a two-stage rotary pump, but such a pump tends to lose its efficiency during prolonged use, particularly if the pump oil becomes fouled with water vapor. Loss of efficiency does not occur when the rotary pump is preceded by a diffusion pump, provided that the diffusion pump is charged with a fluid that resists oxidation (e.g., silicone, polyphenyl ether) and is protected by a suitable cold trap (Hauck, 1982).

As described, conversions of NH_4^+-N to N_2 are carried out on-line with the mass spectrometer, and the N_2 formed is transferred immediately to the inlet system so that the risk of contamination by air will be minimized. However, off-line conversion is necessary when analyses must be performed using a general-purpose mass spectrometer or an instrument equipped with an automated inlet manifold. In such cases, the apparatus illustrated in Fig. 6 must be modified to include a Toepler pump for collection of N_2 samples in sealed tubes or vessels with high vacuum stopcocks. DesMarais and Hayes (1976) have described a simple device for opening sealed glass tubes under vacuum, which allows convenient transfer of gas samples to the mass spectrometer.

In assembling the conversion apparatus, care should be taken to minimize the volume occupied by N_2 during transfer to the mass spectrometer inlet system, so that the transfer will be nearly complete. This is particularly important with viscous inlets of limited expansion volume (e.g., <20 ml), and, in such cases, a smaller sample vial may be required, along with a capillary tube for delivery of LiOBr (Porter and O'Deen, 1977).

Before use, the shell vials should be inspected to eliminate any that are cracked or are too large in diameter to be inserted into the O-ring fitting. The vials recommended are inexpensive enough to be discarded after use, but, if desired, they can be cleaned for reuse by overnight soaking in an acid bath (Vanden Heuvel and Giamalva, 1988).

To avoid contamination of the conversion apparatus by NH_3 liberated upon treatment of the NH_4^+-salt sample with alkaline hypobromite, which can lead to cross-contamination of samples, Ross and Martin (1970) recommended that the sample vial be frozen in dry ice–acetone during the addition of LiOBr. This practice is followed in the procedure described, except that liquid N_2 is used instead of dry ice–acetone. Freezing is more efficient with liquid N_2 than with dry ice–acetone, but the vial must be warmed to allow conversion of NH_4^+-N to N_2 and then refrozen to remove water and hypobromite vapors before transfer of the N_2 to the mass spectrometer inlet system. Removal is sufficiently complete that, if the U-trap used for purification of the N_2 is constructed from 3.2-mm OD tubing as specified, it can be kept frozen throughout the working day with no risk of plugging from accumulation of ice. Ross and Martin (1970) suggested that freezing the sample vial with liquid N_2 may lead to isotopic fractionation from adsorption of N_2, but no such difficulty has been observed by the author.

Experience has shown that, occasionally, a sample vial will crack upon refreezing in liquid N_2. To avoid excessive inlet pressure in such cases, some means should be provided to check the pressure of N_2 before admission to the mass spectrometer. A Hg manometer can be used for this purpose if constructed with capillary tubing, but a low-volume pressure transducer with digital display is more convenient.

In addition to being a strong oxidizing agent, alkaline hypobromite is corrosive toward most metals (including stainless steel), so, periodically, or more often during analyses of samples containing excess acid, the O-ring fitting used to connect the LiOBr reservoir and the sample vial should be detached and disassembled for cleaning with dilute acid, DI water, and acetone. Before reassembly, a light coating of high vacuum grease (e.g., Apiezon® N) should be applied to the O-rings. A similar coating will facilitate operation of the stopcock through which evacuations are performed.

D. Isotopic Analysis of N_2

1. Principles

A discussion of the operation of an isotope ratio mass spectrometer for isotopic analysis of N_2 can be found in Section II.A.

The mass spectrometer measures a ratio of the intensities of the currents produced by two or more of the ion beams, $^{28}N_2^+$, $^{29}N_2^+$, and $^{30}N_2^+$, from which the isotopic abundance, normally expressed as atom% ^{15}N, must be calculated. Several equations may be used for this calculation, depending on the ratio or ratio difference measured (see Section III.D.4).

2. Materials Required

a. Special apparatus: Mass Spectrometer

b. Reagent: Reference N_2

A suitable reference gas may be produced by passing ultra-high purity grade compressed N_2 through a liquid N_2 trap to remove water vapor or by removing water vapor, CO_2, and O_2 from ambient air as described in Chapter 7. Transfer the gas to an evacuated expansion chamber of a dual-inlet system or to an evacuated reservoir for connection to a single-inlet system. Alternatively, generate reference N_2 as needed by hypobromite oxidation of a small amount of reagent-grade $(NH_4)_2SO_4$ of known ^{15}N content.

3. Description of Procedure

With sample N_2 in the expansion volume of the inlet system, adjust the pressure as required, admit the gas to the analyzer assembly, and determine its isotope ratio. Following the analysis, evacuate the expansion volume using the inlet pumping system of the mass spectrometer in preparation for the next sample. Calibrate the mass spectrometer with reference N_2.

The exact procedure for carrying out the isotope ratio analysis depends on the type of mass spectrometer employed, and complete description is beyond the scope of the present discussion. For such information, the reader is referred to the literature supplied by the manufacturer of the mass spectrometer.

4. Calculation of Results

During hypobromite oxidation of NH_4^+, ^{14}N and ^{15}N atoms are distributed randomly among the N_2 molecules formed, such that the relative proportions of the three isotopic species ($^{28}N_2$, $^{29}N_2$, $^{30}N_2$) can be expressed in terms of the atom fraction of ^{15}N in the NH_4^+ oxidized ($^{15}A_N$) according to the following binomial distribution:

$$^{28}N_2 \text{ produced/total } N_2 \text{ produced} = (1 - {}^{15}A_N)^2 \tag{2}$$

$$^{29}N_2 \text{ produced/total } N_2 \text{ produced} = 2\,{}^{15}A_N(1 - {}^{15}A_N) \tag{3}$$

and

$$^{30}N_2 \text{ produced/total } N_2 \text{ produced} = ({}^{15}A_N)^2 \tag{4}$$

Because the reaction is carried out in the absence of air, the sample of N_2 analyzed with the mass spectrometer consists of an equilibrium mixture of these isotopic species, and $^{15}A_N$, or atom% ^{15}N (i.e., $100\,{}^{15}A_N$), may be calculated directly from measurement of a single isotopic ratio or ratio difference. Table II shows several expressions that can be used to perform this calculation. These expressions were derived by algebraic manipulation of appropriate combinations of Eqs. (2)–(4) to give the measured ratio. Calculations should be based on ratio differences whenever analyses are performed using a dual-inlet system. This approach may also be employed for analyses with a single-inlet system, provided that reference N_2 is introduced [from a separate reservoir or by hypobromite oxidation of $(NH_4)_2SO_4$ of known ^{15}N content] at regular intervals between samples and that all analyses are performed at the same inlet pressure. The ratio difference technique has the advantage over measuring absolute ratios that background correction of ion currents is unnecessary, and any consistent error in the ratio measurements will be canceled.

Isotope ratio analyses of N_2 are normally performed on samples containing <5 atom% ^{15}N. In such cases, the mole fraction of $^{29}N_2$ is much larger than that of $^{30}N_2$, and highest accuracy and precision are achieved by measuring an isotopic ratio that contains $^{29}N_2$ in the numerator, usually $^{29}N_2/^{28}N_2$ or $^{29}N_2/(^{28}N_2 + {}^{30}N_2)$. At higher ^{15}N enrichments, the ion current at *m/e* 30 can be measured accurately, and atom% ^{15}N is determined by measuring a ratio with $^{30}N_2$ in the numerator, such as $^{30}N_2/^{28}N_2$ or $^{30}N_2/(^{28}N_2 + {}^{29}N_2)$.

Table II
Expressions for Calculating Atom% ^{15}N from Various Isotopic Ratios and Ratio Differences

Ratio measured (r)	Nominal atom% ^{15}N	Expression to multiply by 100 for calculating atom% ^{15}N from:	
		Ratio[a]	Ratio difference[b]
$^{29}N_2/^{28}N_2$	<5	$r/(2 + r)$	$(\Delta r + K_1)/(2 + \Delta r + K_1)$
$^{30}N_2/^{28}N_2$	≥5	$\frac{-2r + (4r)^{1/2}}{2 - 2r}$	$\frac{-2\Delta r - 2K_2 + (4\Delta r + 4K_2)^{1/2}}{2 - 2\Delta r - 2K_2}$
$^{29}N_2/(^{28}N_2 + {}^{30}N_2)$	<5	$\frac{2r + 2 - (4 - 4r^2)^{1/2}}{4r + 4}$	$\frac{-2 - 2\Delta r - 2K_3 + (4 - 4\Delta r^2 - 4K_3^2 - 8K_3\Delta r)^{1/2}}{-4 - 4\Delta r - 4K_3}$
$^{30}N_2/(^{28}N_2 + {}^{29}N_2)$	≥5	$[r/(1 + r)]^{1/2}$	$[(\Delta r + K_4)/(1 + \Delta r + K_4)]^{1/2}$

[a] Background correction of measured ion currents is required. If measurements are made using multiple collectors, correction must be made for the resistances associated with the electrometer heads.

[b] $\Delta r = r_{\text{sample}} - r_{\text{reference}}$. $K_1 \equiv 2C/(1 - C)$. $K_2 \equiv C^2/(1 - C)^2$. $K_3 \equiv 2C(1 - C)/[C^2 + (1 - C)^2]$. $K_4 \equiv C^2/[(1 - C)^2 + 2C(1 - C)]$. C is the atom fraction of ^{15}N in the reference N_2 (0.003663 for ambient air). If measurements are made using multiple collectors, correction must be made for the resistances associated with the electrometer heads. For measurements with a dual-inlet system, a zero enrichment factor should be determined by equilibrating the same sample between both sides of the inlet system. This factor, calculated as the difference between ratios measured for sample and reference inlets, is subtracted from all ratio difference measurements (i.e., $\Delta r = r_{\text{sample}} - r_{\text{reference}} -$ zero enrichment factor).

Nitrogen isotopic composition is sometimes expressed in terms of atom% ^{15}N excess, which is calculated by subtracting the background concentration of ^{15}N (conventionally, 0.3663 atom%) from an experimentally determined value of atom% ^{15}N. This practice has been criticized on the grounds that there is variability in the ^{15}N content of natural materials, which leads to uncertainty in the interpretation of data (Hauck, 1982). To avoid such difficulty, the value used for the background concentration of ^{15}N must always be specified when data are reported in terms of atom% ^{15}N excess.

5. Comments

Serious error can arise if the sample of N_2 is contaminated by air before isotope ratio analysis with the mass spectrometer. The most likely cause of contamination is leakage of air into the hypobromite conversion apparatus after oxidation of NH_4^+-N to N_2. A correction for air leakage has sometimes been applied to ratio measurements based on the intensity of the O_2 peak at *m/e* 32 (see Bremner, 1965d), but the validity of this practice is clearly questionable in view of the fact that O_2 is produced during decomposition of hypobromite (see Section III.C.1). Air leakage can also be estimated by measuring the Ar peak at *m/e* 40; however, the usefulness of this technique is limited by the low Ar content of air (≃40 μmol liter^{-1}). In view of the difficulties involved, correction of isotopic ratio data for air leakage is not recommended. Rather, precautions should be taken to prevent contamination by air during preparation and transfer of gas samples. This requires frequent leak-testing. Ideally, leak-testing is accomplished by measuring the intensity of the peak at *m/e* 32 for every sample analyzed, as suggested by Hauck (1982). By experience, an upper limit is determined for measurements of normal O_2 content. Excessive O_2 indicates air leakage or the presence of substances in the NH_4^+ sample that react with hypobromite to liberate O_2, such as excess acid (Hauck, 1982). Leakage of air is confirmed by an elevated O_2 level during isotope ratio analysis of N_2 generated from reagent-grade $(NH_4)_2SO_4$. In such cases, isotope ratio analyses for all samples with high O_2 content are rejected, and no further analyses are performed until the air leak has been located and eliminated.

The amount of N needed for isotope ratio analysis varies considerably, depending on the type of mass spectrometer used. Samples containing 0.5 mg NH_4^+-N can be analyzed with many modern isotope ratio mass spectrometers, whereas most older instruments require at least 1 mg N. Samples containing less N than required can be analyzed by addition of a known quantity of $(NH_4)_2SO_4$ of known ^{15}N content, with calculation of results by an isotope dilution equation (see Hauck, 1982). The isotope dilution technique is also useful for analyses of highly depleted (<0.01 atom% ^{15}N) or enriched (>5 atom% ^{15}N) samples. Addition of natural abundance NH_4^+-N to highly depleted samples produces a measurable ion current at *m/e* 29. A similar addition to highly enriched samples reduces measurement errors caused by the nonlinear amplification of ion currents or by incomplete evacuation of N_2 from a previous sample (memory). The latter problem can be especially serious if the mass spectrometer is equipped with ion pumps.

Isotope ratio measurements tend to increase with decrease in inlet pressure (Mulvaney and Liu, 1991). This can lead to error in analyses of small samples with a single-inlet system, unless calibration with reference N_2 has been performed at a similar pressure. Such difficulty does not arise with a dual-inlet system, because analyses of sample and reference N_2 are always carried out at the same pressure.

Because atmospheric N_2 is a reliable standard for N isotopic analyses (Mariotti, 1983), ambient air is the preferred source of reference N_2 for N isotopic analyses of ^{15}N-depleted, natural-abundance, and low enrichment (<5 atom% ^{15}N) samples. However, water vapor and O_2 must be removed before use, and this requires a special purification apparatus, such as the units described in Chapter 7. Purification is simplified considerably through the use of commercially available compressed N_2, but the ^{15}N content may differ slightly from that of atmospheric N_2. Regardless of the source of reference N_2, the accuracy of isotope ratio analyses should be checked periodically using N_2 generated from a small amount of reagent-grade $(NH_4)_2SO_4$ that has been standardized relative to an ^{15}N reference material, such as the $(NH_4)_2SO_4$ intercomparison standards available from the International Atomic Energy Agency, Wagramerstrasse 5, A-1400 Vienna, Austria.

IV. SAMPLE PREPARATION TECHNIQUES FOR AUTOMATED N ISOTOPE ANALYSIS

A. General Principles

Special techniques are required in the preparation of samples for isotope ratio analysis by the automated mass spectrometers described in Section II.F. For automated analyses by the Rittenberg technique, via ARA-MS, an NH_4^+-salt sample is required that contains between 20 and 150 μg N, and final drying must be carried out with the sample in a well of a plastic microplate. In analyses by ANCA-MS, all forms of N are converted directly to N_2, and the only requirements for samples are that they be in a dried form in a sealed Sn capsule and contain between 20 and 150 μg N (the amount of N required depends on the particular system used and several operating parameters). Solid materials may be analyzed directly by ANCA-MS, but the material must be highly homogeneous to obtain a representative sample, and this requires special grinding techniques.

B. Automated Rittenberg analysis

1. Principles

Isotope ratio analyses by ARA-MS involve the same three-step procedure employed in conventional N isotope analyses (Section III.A), the only difference being that steps 2 and 3, involving hypobromite oxidation of NH_4^+-N to N_2 and isotopic analysis of the N_2, are performed automatically rather than manually. Conversion of labeled N to NH_4^+-N may be carried out as described in Section III.B, with transfer of an appropriate aliquot of the concentrated distillate to a plastic sample tray for final evaporation in a low-temperature oven or dehydrator. However, Kjeldahl digests may also be diffused by the method described in Section IV.D.3.b, and this is usually more convenient than steam distillation, particularly when an autoanalyzer is available for colorimetric determination of NH_4^+-N in the digest.

2. Materials Required

a. Special apparati In addition to items 1 through 5 specified in Section III.B.2.a, the following are required.

1. Plastic sample trays. The trays required are Microtiter® disposable plates (catalog no. 001-010-2301; "U" wells; sterile) manufactured by Dynatech Laboratories (14340 Sullyfield Circle, Chantilly, Virginia 22021). A tray has 96 wells, each with a capacity of 0.3 ml.
2. Low-temperature oven or food dehydrator.

b. Reagents Reagents 1–10 specified in Section III.B.2.b are required.

3. Description of Procedure

Carry out digestion as described in Section III.B.3.a, and steam-distill the digest with NaOH as described in Section III.B.3.b. Following titration of the distillate, add 0.2 ml 0.4 *M* H_2SO_4, and evaporate to dryness (85–90°C). Then dissolve the residue in 1–3 ml DI water, and transfer an aliquot (to 0.3 ml) containing 20–150 μg (preferably 50–150 μg) NH_4^+-N to a well of a sample tray. Evaporate to dryness by heating the tray at 50–60°C in an oven or dehydrator. After drying, apply Parafilm® to the top of the tray, and secure it in place with rubberbands. For shipment to a service laboratory (Isotope Services, 329 Potrillo Drive, Los Alamos, New Mexico 87544; or N-15 Analysis Service, Department of Agronomy, University of Illinois, Turner Hall, 1102 South Goodwin Avenue, Urbana, Illinois 61801), trays should be packaged in a well-padded box.

Table III
Amount of H_2SO_4 Recommended for Collection of NH_4^+-N for Isotope Ratio Analysis by ARA-MS.

NH_4^+-N in distillate (μg)	H_2SO_4 for collection	
	Volume (ml)	Concentration (*M*)
20–300	5	0.0025
300–600	5	0.005
600–1200	5	0.01
1200–2400	5	0.02
2400–4800	5	0.04

4. Comments

Although isotope ratio analysis by ARA-MS can be performed on samples containing 20 μg N, analysis of distillates collected in H_3BO_3-indicator solution is complicated by the fact that the wells in a sample tray can accommodate the residue from no more than about 0.5 ml H_3BO_3-indicator solution. This represents one-tenth of the volume of indicator used in collection of distillates; therefore, the distillate must contain at least 200 μg N to obtain a suitable sample for isotope ratio analysis. An alternative is to perform two distillations on each digest or on duplicate digests, with collection of the first distillate in H_3BO_3-indicator solution for quantitative determination of NH_4^+-N, followed by collection of the second distillate in dilute H_2SO_4 for concentration and isotope ratio analysis. To avoid addition of excess acid, which interferes with hypobromite oxidation of NH_4^+ through formation of Br_2 (see Section III.C), the amount of H_2SO_4 used should not exceed the recommendations in Table III.

Because of their low N content, serious contamination can occur if samples are exposed to atmospheric NH_3 during final drying in a plastic tray. To minimize contamination, the oven or dehydrator used should be located away from any source of ambient NH_3 (see Sections III.B.1.d and III.B.4.c), and there should be no external flow of forced air. To avoid deforming the sample trays, which precludes processing by ARA-MS, final drying should never be done with a hot plate, but only as specified, at a temperature not exceeding 60°C.

To check the accuracy of the analyses, samples of known ^{15}N content should be included in each sample tray.

If collected in dilute H_2SO_4 (Table III), samples may be submitted to Isotope Services in screw-cap vials as a concentrated distillate containing at least 0.1 mg NH_4^+-N ml^{-1} (<50 atom% ^{15}N) or as the dried residue from such a distillate. Isotope ratio analyses are performed on duplicate aliquots of each sample and are repeated if the results do not agree to within 0.8%.

C. Direct Combustion

1. Principles

In addition to the Kjeldahl procedure, total N analyses can be performed by the Dumas technique, which involves heating the sample with CuO to convert N to N_2 and N oxides (mainly N_2O); the N oxides are subsequently reduced to N_2 with hot Cu (Bremner and Mulvaney, 1982). Use of the Dumas technique has traditionally been limited by incomplete recovery of oxidized N (e.g., NO_3^-) at the temperatures employed for combustion (550–650°C). This problem was overcome by development of the ANCA, in which complete oxidation is achieved through flash combustion (≃1700°C) in the presence of a catalyst (e.g., Cr_2O_3); the combustion products are swept over Cu at 600°C and then purified chromatographically. The ANCA has been successfully interfaced with quadrupole and magnetic sector mass spectrometers for automated analysis of total N and ^{15}N (see Section II.F), and commercial systems have become available that consist of an ANCA coupled to a compact magnetic sector mass spectrometer. A manual method for direct combustion is described in Chapter 6, Section II.C.

2. Materials Required

a. Special apparati

1. Grinding apparatus (for solid samples). Grinding can be accomplished by rotation of glass bottles that contain stainless-steel rods of various diameters along with the sample to be ground. The 118-ml (4-oz.) straight-sided, round bottle available from Fisher Scientific (catalog no. 03-320-3E) is satisfactory. For grinding, 20 3.2- × -66.7-mm, 12 4.8- × -66.7-mm, and 6 6.4- × -66.7-mm stainless-steel rods are placed in each jar (Kelley and Cannon, 1990). Rotation of the bottles (100–130 rpm) may be accomplished with a roller mill (Harris and Paul, 1989), but the conveyor belt assembly described by Kelley and Cannon (1990) is more convenient for large numbers of samples, because up to 55 samples can be ground concurrently. Samples must be dried before grinding; moreover, plant materials should be ground to <1 mm (or finer) with a rotary mill. With the method of Kelley and Cannon (1990), a 40-ml sample is ground for 8–12 hr, which is sufficient to reduce soil and most plant materials to <150 μm. The ground sample may be stored indefinitely in the same bottle used for grinding. Before reuse, the stainless-steel rods are washed with detergent, rinsed with DI water, soaked in dilute acid (e.g., 0.1 *M* HCl) for 5–10 min, rinsed with DI water, and dried.
2. Freeze-dryer (for liquid samples).
3. Tin sample containers. The 8- × -5-mm containers available from Conroy Scientific (155 Rochester Street, Costa Mesa, California 92627; catalog no. 4001) are acceptable. Before use, the containers should be washed (CCl_4, acetone, and DI water) and then dried in an oven.
4. Microbalance, readable to 0.01 or 0.001 mg.

b. Reagents No reagents are required in the preparation of solid or liquid samples for analyses by ANCA-MS.

3. Description of Procedures

a. Solid samples Place an accurately weighed (to 0.01 mg) sample of finely ground soil or plant material containing approximately 100 μg N (20–60 mg soil, 2–15 mg plant material) in a Sn sample container. While holding the container with a forceps, crimp the mouth closed with a broad-tipped forceps. Then compress the container into a compact cylinder and transfer it to an autosampler carousel.

b. Liquid samples Pipette an aliquot of the solution under analysis containing approximately 100 μg N into a Sn sample container. Evaporate the solution to dryness in a freeze-dryer. Then close the mouth of the sample container with a broad-tipped forceps, compress it into a compact cylinder, and transfer it to an autosampler carousel.

4. Comments

Some plant materials, particularly fibrous stems and roots, are difficult to grind finely. Such materials can usually be ground to sufficient fineness by the technique described, provided that grinding is performed for at least 12 hr and that the raw material is first chopped to <1 mm in a rotary mill. The rotary mill must be carefully cleaned between samples to prevent cross-contamination. A highly effective alternative is to carry out grinding at the temperature of liquid N_2 (−195.8°C), at which materials become brittle. An apparatus expressly designed for cryogenic grinding is available from Spex Industries (3880 Park Avenue, Edison, New Jersey 08820). With this apparatus, grinding is readily accomplished to <150 μm, at a rate of up to eight samples per hour. However, sample volume cannot exceed 3 ml, and cleaning is required after every sample to prevent cross-contamination.

Reference standards for calibration of total N and ^{15}N must be included at the beginning of a run and at regular intervals thereafter, normally following a series of 6–12 samples. Reagent-grade $(NH_4)_2SO_4$ may be used as the reference material, but the N content is sufficiently high that weighing must be done to 1 μg with a microbalance in order to obtain a reliable weight of material that contains 100 μg N. Weighing to 1 μg is also necessary when the $(NH_4)_2SO_4$ is dispensed from solution, because the precision of microliter pipettes is inadequate for calibration of total N determinations by ANCA-MS (Harris and Paul, 1989). To make weighing more convenient, a reference material having a lower N content than $(NH_4)_2SO_4$ may be used, such as primary-standard grade THAM®. Use of THAM® allows weighing to be performed with an analytical balance readable to 0.01 mg.

If desired, liquid samples may be absorbed into an inert support such as Chromosorb®, which eliminates the need for freeze-drying. However, care must be taken to ensure that the sample is not exuded during compression of the Sn container. Moreover, the support material contributes to accumulation of ash in the combustion tube, which must be removed periodically to ensure complete combustion (Preston and McMillan, 1988).

For shipment to a service laboratory offering analyses by ANCA-MS (e.g., Isotope Services, 329 Potrillo Drive, Los Alamos, New Mexico 87544), the Sn sample containers may be transferred to individual screw-cap vials. Usually, however, weighing and loading of solid samples into the Sn containers is carried out at the service laboratory, in which case shipment of finely ground samples (0.1 g plant material, 1 g soil) is conveniently accomplished using polyethylene vials.

D. Diffusion of NH_4^+-N

1. Principles

Diffusion techniques are an alternative to steam distillation for separation and collection of NH_4^+-N (Hauck, 1982), and these techniques have special value in the preparation of samples for automated mass spectrometers, because only microgram quantities of N are required. Recent work by Brooks *et al.* (1989) has led to the development of a diffusion procedure for automated isotope ratio analysis of inorganic N in soil extracts. With this procedure, the extract is treated with a mild alkali (MgO) and a reducing agent (Devarda's alloy) to convert $(NH_4^+ + NO_3^-)$-N to NH_3-N. Conversion is carried out at room temperature in a disposable specimen container, and the NH_3-N evolved is collected as NH_4^+-N in an acidified disk cut from a glass fiber filter with a paper punch. Although developed for diffusion of 2 *M* KCl extracts of soil, the method of Brooks *et al.* (1989) has application to other types of samples, including 0.5 *M* K_2SO_4 extracts and natural water samples. A simple modification allows diffusion of Kjeldahl digests with NaOH (Liu and Mulvaney, 1992a).

Isotope ratio analysis of the diffused NH_4^+-N can be performed by ARA-MS after addition of DI water to the glass disk, followed by transfer of an aliquot to a sample tray, but a simpler and more convenient technique is to perform the analysis on the disk itself after addition of hydrofluoric acid (HF), which degrades the disk without affecting the NH_4^+ it contains or the plastic sample tray (Liu and Mulvaney, 1992b).

For analyses by ARA-MS, the glass fiber disk is acidified with dilute H_2SO_4. When analyses are to be performed by ANCA-MS, acidification is accomplished using a solution of $KHSO_4$, because H_2SO_4 would corrode the Sn container in which combustion is carried out.

2. Materials Required

a. *Special apparati*

1. Spectrophotometer or autoanalyzer for colorimetric determination of NH_4^+ and NO_3^- in soil extracts or of NH_4^+ in Kjeldahl digests.
2. Diffusion apparatus. The unit described by Brooks *et al.* (1989) consists of a 118-ml specimen container (e.g., Premium Plastics no. 4653; available as catalog no. 42354 from General Medical Corporation, 775 Belden Avenue, Suite F, Addison, Illinois 60101), a 62-mm piece of stainless-steel wire (cut from 0.8-mm-diameter 316L stainless-steel welding rod), and a 7-mm-diameter glass fiber disk. The glass disk is cut with a paper punch from a circle or sheet of Whatman® GF/D glass fiber filter material.
3. Shell vials, used to introduce NaOH in diffusion of Kjeldahl digests. Kimble No. 60930L-12 (12 mm OD, 35 mm long) is satisfactory.
4. Plastic sample trays (for analyses by ARA-MS; see Section IV.B.2.a) or Sn sample containers (for analyses by ANCA-MS; see Section IV.C.2.a). Before use, the mouths of the solvent-washed Sn containers should be widened with a forceps.
5. Desiccator, used to carry out drying of glass disks following diffusion.

b. *Reagents*

1. Magnesium oxide powder (for diffusion of soil extracts).
2. Devarda's alloy (for diffusion of soil extracts). A satisfactory alloy is produced by E. Merck (no. 5341; available from EM Science, P.O. Box 70, 480 Democrat Road, Gibbstown, New Jersey 08207).
3. Sodium hydroxide solution, approximately 20 *M* (for diffusion of Kjeldahl digests). To 800 ml DI water in a 1-liter volumetric flask, add 800 g

NaOH pellets, and swirl the flask until the alkali is dissolved. Cool the solution, and bring the volume to 1 liter by adding DI water.

4. Sulfuric acid (1 *M*) or $KHSO_4$ (2.5 *M*). For analyses by ARA-MS, add 11 ml concentrated H_2SO_4 to approximately 150 ml DI water in a 200-ml volumetric flask, bring to volume with DI water, and mix thoroughly. For analyses by ANCA-MS, dissolve 68.1 g $KHSO_4$ in approximately 180 ml DI water in a 200-ml volumetric flask, dilute the solution to volume, and mix thoroughly.
5. Anhydrous calcium sulfate. Indicating Drierite® is satisfactory.
6. Hydrofluoric acid, concentrated (28.9 *M*). This reagent is required only if isotope ratio analyses are to be performed by ARA-MS.
7. Potassium hydroxide pellets. This reagent is required only if isotope ratio analyses are to be performed by ARA-MS.

3. Description of Procedures

a. Diffusion of soil extracts[6] By colorimetry or other suitable technique (see Keeney and Nelson, 1982), determine the concentrations of NH_4^+ and NO_3^- in a 2 *M* KCl soil extract [prepared as described by Keeney and Nelson (1982)]. Pipet an aliquot (5–60 ml) that contains 50–150 μg (NH_4^+ + NO_3^-)-N into a specimen container, and add a 4-mm-diameter acid-washed glass bead. Pierce a glass fiber disk with a 62-mm piece of stainless-steel wire, approximately center the disk on the wire, and treat it with 10 μl of either 1 *M* H_2SO_4 (for analysis by ARA-MS) or 2.5 *M* $KHSO_4$ (for analysis by ANCA-MS). For storage until needed, place the wire with the acidified disk across the top of a 100-ml beaker. Then use calibrated spoons to add 0.1 g Devarda's alloy and 0.2 g MgO to the sample in the specimen container. Immediately position a wire with an acidified disk across the top ledge of the specimen container, and seal the container by firmly attaching a screw cap. Thoroughly bring the MgO and Devarda's alloy into suspension by sliding the specimen container with a circular motion on a laboratory benchtop. Do this without lifting the container off the bench; otherwise, the basic mixture may come into contact with the glass disk and neutralize its acidity. After mixing for approximately 20 sec, allow the container to stand at room temperature (≥20°C) for 6 days without shaking.

For isotope ratio analysis of the diffused NH_4^+-N by ARA-MS, remove the wire with the glass disk from the specimen container, and transfer the disk to a well of a sample tray using the wire to position the disk in the mouth of the sample well, drawing the wire across the top of the tray so as to leave the disk in the sample well, and then pushing the disk into the bottom of the well with the wire. Following transfer, treat the disk with 10 μl concentrated HF, and dry it by placing the sample tray over anhydrous $CaSO_4$ in a desiccator with a small beaker of KOH pellets to absorb HF vapor. When drying is complete (after 1–3 days, the residue from the digested disk will form a thin white coating at the bottom of the sample well), apply Parafilm® to the top of the tray for shipment to a service laboratory (see Section IV.B.3).

For analysis by ANCA-MS, remove the wire with the disk containing the diffused NH_4^+-N from the specimen container, and dry the disk overnight with the wire inserted into a Styrofoam® block in a desiccator over anhydrous $CaSO_4$. Then remove the wire from the desiccator, and transfer the glass disk to a Sn sample container by pushing it off the wire with a bent paper clip. Using forceps, compress the Sn container around the disk, but take care to avoid touching the disk with the forceps. Transfer the encapsulated disk to an autosampler carousel for analysis, or place it in a screw-cap vial for shipment to a service laboratory (see Section IV.C.4).

b. Diffusion of Kjeldahl digests[7] Following Kjeldahl digestion of a sample containing approximately 1 mg N by the procedure described in Section III.B.3.a, dilute the digest to 25 ml with DI water (if an Al block digester has been employed, dilution may be conveniently accomplished using a digestion tube marked to indicate a volume of 25 ml), and mix thoroughly (e.g., by vortexing). By colorimetry or other suitable technique, determine the concentration of NH_4^+-N in the diluted digest. Pipet an aliquot that contains 50–150 μg NH_4^+-N into a specimen container, add sufficient DI water to bring the volume to 20 ml, and insert a vial containing 2 ml 20 *M* NaOH such that it stands in an upright position and is in contact with the wall of the specimen container. Pierce a glass fiber disk with a 62-mm piece of stainless-steel wire, approximately center the disk on the wire, and treat it with 10 μl 1 *M* H_2SO_4. Position the wire across the top ledge of the specimen container, and seal the container by firmly attaching a screw cap. Then slide the container in a circular pattern on a laboratory benchtop to overturn the vial and swirl the contents, taking care that the alkaline mixture does not splash upward and neutralize the acidified glass fiber disk. After mixing for approximately 20 sec, allow the container to stand at room temperature (≥20°C) for 6 days without shaking. For isotope ratio analysis of the diffused NH_3-N by ARA-MS, treat the glass disk with HF in a plastic sample tray as described in Section IV.D.3.a, and dry for 1–3 days in a desiccator over anhydrous $CaSO_4$.

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[6] After Brooks *et al.* (1989) and Liu and Mulvaney (1992b,c).

[7] After Liu and Mulvaney (1992a,b).

4. Comments

The procedures described are modifications of the diffusion method developed by Brooks *et al.* (1989). These procedures are simple and convenient; nevertheless, they should be thoroughly evaluated in one's own laboratory before routine use is undertaken. This is particularly important in diffusion of soil extracts, because serious error has been observed under some conditions (Jensen, 1991; Liu and Mulvaney, 1992c). Evaluation can be accomplished through a comparison involving ^{15}N analyses by diffusion and by conventional steam distillation and concentration techniques, but the recovery of diffused N should also be checked, because serious error can arise from isotope fractionation if diffusion is incomplete (MacKown *et al.*, 1987). The latter quantity is readily obtained by a colorimetric determination of NH_4^+-N in the diffusion disk (Liu and Mulvaney, 1992c).

The 6-day period recommended for diffusion is based on work by Brooks *et al.* (1989) showing that shorter periods are insufficient for complete liberation of NH_4^+-N from soil extracts treated with MgO and Devarda's alloy. The rate of diffusion is necessarily limited by the diameter of the specimen container, which determines the surface-to-volume ratio of the sample, but some reduction in the diffusion period can be achieved by heating. For example, Kelley *et al.* (1991) obtained complete diffusion of NH_4^+-N in 5 days at 55°C.

When soil extracts are diffused as described, $(NH_4^+ + NO_3^- + NO_2^-)$-N is recovered. In most cases, the amount of NO_2^- is negligible, and the N recovered by diffusion represents $(NH_4^+ + NO_3^-)$-N. Brooks *et al.* (1989) suggested that NH_4^+-N and NO_3^--N could be recovered separately by carrying out two 6-day diffusions with separate acid traps, the first involving addition of MgO to liberate NH_4^+-N, with subsequent addition of Devarda's alloy to recover NO_3^--N. However, recent work by Jensen (1991) and Liu and Mulvaney (1992c) indicates that the sequential approach is unsatisfactory, because ^{15}N analyses of NO_3^--N were increased substantially by a prior diffusion of NH_4^+-N having a higher content of ^{15}N. An alternative is to diffuse duplicate aliquots of each soil extract, using MgO with and without Devarda's alloy to recover NH_4^+-N and $(NH_4^+ + NO_3^-)$-N, which allows the ^{15}N content of NO_3^- to be calculated by an isotope-dilution equation (Liu and Mulvaney, 1992c). If necessary, an additional diffusion can be carried out after treatment of the soil extract with sulfamic acid to remove NO_2^--N, in which case an ^{15}N content can be determined for NH_4^+, NO_3^-, and NO_2^- (Liu and Mulvaney, 1992c).

Either light or heavy MgO is satisfactory as the alkaline reagent in diffusion of soil extracts, and it may be used without being ignited to remove $MgCO_3$ (Liu and Mulvaney, 1992c). The Devarda's alloy specified has a lower N content than the alloys normally employed with steam distillation procedures (Keeney and Nelson, 1982), and this is important to avoid excessive levels of background N, which can vitiate ^{15}N analyses by diffusion (Liu and Mulvaney, 1992c). The alloy specified is supplied in the form of a finely ground powder (particle size <0.2 μm), and further grinding before use is unnecessary to increase reactivity (Liu and Mulvaney, 1992c). However, thorough mixing with a glass bead is essential to ensure complete reduction of NO_3^- to NH_4^+.

The procedure described for diffusion of Kjeldahl digests was developed for ^{15}N determinations by ARA-MS, but it is readily adapted for use with ANCA-MS by acidifying the glass fiber disk with 10 μl 2.5 *M* $KHSO_4$ instead of H_2SO_4. The need for such use would normally not arise, in that analyses by ANCA-MS utilize Dumas combustion to convert sample N directly to N_2 (see Sections II.F and IV.C). However, Kjeldahl digestions may be carried out as an additional step (Egsgaard *et al.*, 1989), and this is sometimes useful, as when total ^{15}N analyses must be performed on moist soils or soil extracts or on natural waters.

As described, diffusions are performed using semimicro-Kjeldahl digests, but the same procedure can be employed when digestion is done by a macroversion of the Kjeldahl method (Bremner, 1965a). The amount of NaOH used (2 ml of a 20 *M* solution) is sufficient to neutralize 1 ml 18 M H_2SO_4, which determines a lower limit for the N content of the digest. With the digestion procedure described in Section III.B.3.a, this limit is 250 μg (Liu and Mulvaney, 1992a); however, the addition of NaOH can be increased to diffuse digests containing <250 μg N. For example, by using two vials to introduce 4 ml NaOH, diffusions may be performed with as little as 125 μg N in the digest. Dilution with DI water to 20 ml before neutralization is important, because otherwise the heat produced by reaction of H_2SO_4 and NaOH may damage the specimen container.

If necessary, up to several hundred soil extracts or Kjeldahl digests may be diffused at one time. However, in such cases, diffusions should be initiated individually or with samples subdivided into small batches, so that the acidified glass disks are not exposed to atmospheric NH_3 for more than 10 min before transfer to a specimen container (Brooks *et al.*, 1989).

In the diffusion procedure recommended by Brooks *et al.* (1989) for use with ARA-MS, acidification of the glass disk is accomplished using 10 μl 5 *M* H_2SO_4, on the assumptions that (1) >100 μg N will be diffused and (2) the analysis will be performed on a sample of the diffused NH_4^+-N, obtained by treating the disk with a small volume of water and transferring an aliquot to a well of a sample tray. However, analyses of the disks themselves are complicated by the presence of excess acid, which decomposes the hypobromite used to convert NH_4^+-N to N_2 (see Section III.C).

With the procedures described, this difficulty is avoided by acidifying diffusion disks with 10 μl 1 *M* H_2SO_4, which provides a trapping capacity of 280 μg N. By comparison, the 10 μl 2.5 *M* $KHSO_4$ used in carrying out diffusions for ANCA-MS can trap up to 350 μg N (Brooks *et al.*, 1989).

The specimen containers are inexpensive enough to be discarded after use, and this has the important advantage that cross-contamination error is avoided. However, they may safely be reused if washed with detergent, soaked for at least 1 hr in an acid bath (e.g., 1 *M* H_2SO_4), and then rinsed with DI water (Liu and Mulvaney, 1992c). The pieces of stainless-steel wire may be reused if soaked overnight in an acid bath.

When isotope ratio analyses are to be performed by ANCA-MS, controls should be included to allow correction for isotopic dilution of sample N by natural abundance N derived from the reagents or from ambient NH_3 (Kelley *et al.*, 1991). For analyses by ARA-MS, a colorimetric determination is made of the amount of NH_4^+-N collected by diffusion of controls (Liu and Mulvaney, 1992c). Alternatively, diffusions are performed using samples of known ^{15}N enrichments, so that the amount of background N can be calculated by an isotope-dilution equation (Hauck, 1982).

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3

Optical Emission Spectrometry

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I. INTRODUCTION

A. Development of the Optical Emission Method

Although the precision of mass spectrometry is unequaled for ^{15}N analysis, its high cost and complexity stimulated research into possible alternative spectroscopic methods based on absorption or emission of electromagnetic radiation by N_2. Isotope effects are found in vibrational (infrared) spectra, but vibrational transitions are forbidden in symmetrical diatomic molecules without a permanent dipole moment, such as N_2. Electronic transitions, which occur in the visible (VIS) and ultraviolet (UV) regions, are not affected by the isotopic composition of the molecule. Information on vibrational levels can be obtained indirectly, however, from the vibrational substructure of electronic transitions, and this phenomenon is the basis of the optical emission method of ^{15}N analysis. Nitrogen gas at low pressure in a discharge tube is excited to higher electronic levels by absorbing energy from an external radio-frequency (RF) source (the electrodeless discharge) and emits radiation in the VIS-UV region. The ^{15}N enrichment is determined from the relative intensities of the $^{14}N_2$, $^{14}N^{15}N$, and $^{15}N_2$ lines of the $2 \rightarrow 0$ vibrational transition of the $C(^3\Pi_u) \rightarrow B(^3\Pi_g)$ electronic transition in the near-UV region (this electronic band is also called the second positive group).

Nitrogen-15 was in fact first discovered from such isotope effects in the electronic emission spectrum of NO (Naudé, 1929). Herzberg (1930) first studied the bands of $^{15}N^{14}N$ in the second positive group of N_2 and

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estimated the natural abundance of ^{15}N. The analytical method was initially developed by Clusius and Becker (1947) and then refined by Hoch and Weisser (1950), Hürzeler and Hostettler (1955), Broida and Chapman (1958) and Faust (1960). Following this early work, a high level of interest continued, including the development of commercial analyzers. Analysis of ^{15}N by both optical emission and mass spectrometry has been reviewed by Fiedler and Proksch (1975), Fiedler (1984), Hauck (1982), and Hirschberg and Faust (1985). Middleboe and Johansen (1991) have also recently discussed analysis of nitrogen, carbon, and oxygen isotope ratios by optical emission analysis.

B. Theoretical Background

A population of molecules in a given electronic energy level is distributed in a series of vibrational energy levels (Fig. 1). The energy differences between vibrational levels decrease with increasing vibrational quantum number and also differ slightly for different electronic states. For this reason, transitions between electronic levels display a substructure resulting from transitions in which the vibrational quantum number changes by 0, ±1, ±2, etc. For a given electronic energy level, the proportion of molecules occupying each vibrational level decreases with increasing vibrational quantum number, so that the intensity of transitions decreases with the involvement of higher vibrational states. Figure 2 shows the emission spectrum (at natural abundance) of the $C(^3\Pi_u) \rightarrow B(^3\Pi_g)$ transition of N_2. The vibronic peaks (bandheads) are not symmetrical but have a sharply defined rise on the longer-wavelength side and a trailing edge (or shade) toward shorter wavelengths. The latter is due to the rotational substructure, which in turn underlies each of the vibronic transitions. Spectra showing resolved rotational fine structure in the 2 → 0 transitions of $^{14}N_2$ and $^{14}N^{15}N$ at 297.7 and 298.3 nm, respectively, and also of common impurity gases may be seen in Broida and Chapman (1958), Fiedler and Proksch (1975, pp. 41, 45), Guiraud and Fardeau (1980), and Leicknam *et al.* (1968a).

The choice of bandheads suitable for ^{15}N analysis is limited, due to the decreasing intensity of bands involving higher vibrational levels and variations in the size and sign of the isotope shift (Table I). The transitions with isotope shifts to shorter wavelengths are less suitable for ^{15}N analysis, because the $^{14}N^{15}N$ and $^{15}N_2$ peaks are shifted onto the trailing shoulder of the $^{14}N_2$ peak. Isotope shifts to longer wavelengths (lower energy), as is the case for the 1 → 0 and 2 → 0 bands, move the $^{14}N^{15}N$ and $^{15}N_2$ transitions away from the sharp edge of the $^{14}N_2$ band and also into a region well separated from other bands. Some of the earlier studies used the more intense 1 → 0 transition at 315 nm. The question of which band was most suitable for ^{15}N analysis was examined by Leicknam *et al.* (1968a) and Meier and Müller (1965), who recommended the 2 → 0 transition at 297 nm. Figure 2b,c shows the three bands of $^{14}N_2$, $^{14}N^{15}N$ and $^{15}N_2$ for the 2 → 0 transition at 15 and 60% atom% ^{15}N.

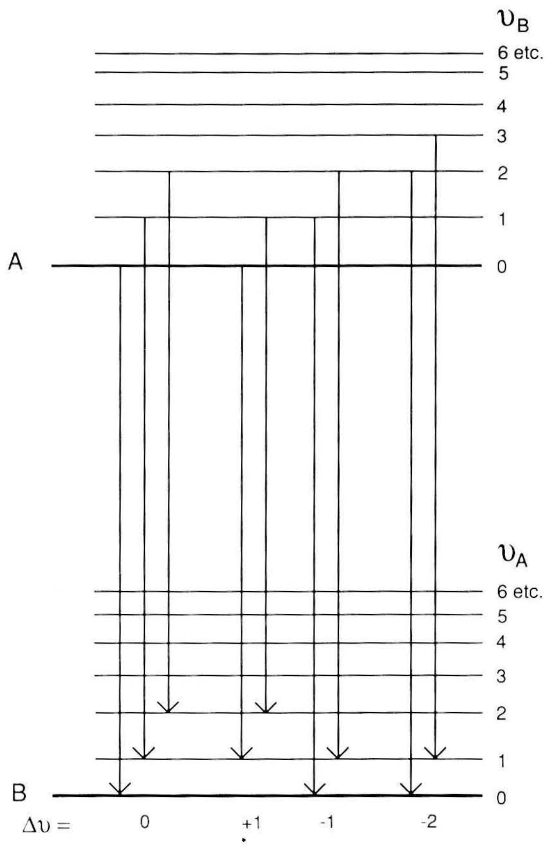

Figure 1 Transitions between two electronic energy levels (A, B). The vibrational quantum number (ν_A, ν_B) changes by $\Delta\nu$.

II. EQUIPMENT REQUIRED

A. For Spectral Analysis

1. Components

Figure 3 shows schematically the basic components of a conventional system for optical emission ^{15}N analysis. The sample of nitrogen gas is

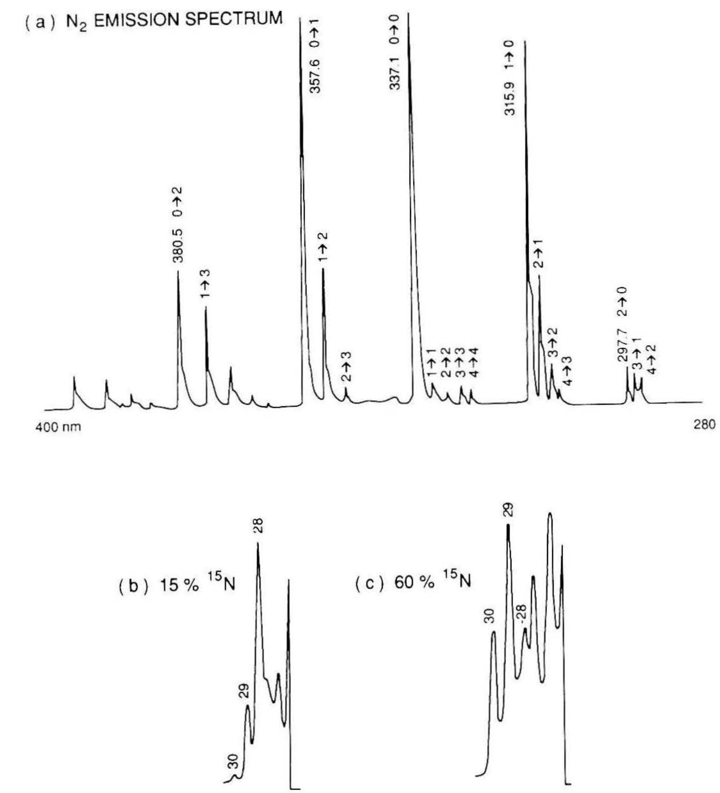

Figure 2 (a) The emission spectrum of N_2 from 280 to 400 nm and isotopic bands for the 2 → 0 transition at 15 (b) and 60 (c) atom% ^{15}N. The spectra were obtained using the new system described in Section II.A.3.

Table I
Bandheads and Isotope Shifts (in nm) for Selected Bands of the Second Positive Group of N_2.

Transition	$^{14}N_2$	$\Delta(^{14}N^{15}N-^{14}N_2)$	$\Delta(^{15}N_2-^{14}N_2)$
2 → 0	297.68	+0.61	+1.18
1 → 0	315.93	+0.36	+0.69
0 → 0	337.13	+0.01	+0.02
0 → 1	357.69	−0.36	−0.78
0 → 2	380.49	−0.84	−1.62
0 → 3	375.54	−0.73	−1.41

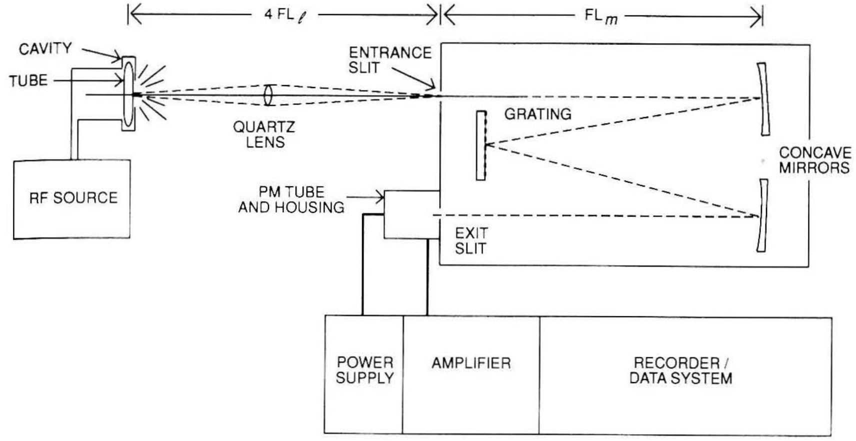

Figure 3 Basic components of an optical emission ^{15}N analyzer. The monochromator is shown with a Czerney–Turner configuration. Some systems include a chopper between the source and entrance slit.

excited by an external RF source. The light emitted by the sample is focused by a quartz lens onto the entrance slit of a grating monochromator. The variation of intensity with wavelength at the exit slit is detected by a photomultiplier (PM) tube and amplified, and this information may be presented directly on a chart recorder or entered into computer memory for time-averaging or other data processing. Discussion of the individual components will be followed by information on instruments that are commercially available or have been reported in the literature. There have been several recent reviews on optical emission analysis (Fiedler and Proksch, 1975; Hauck, 1982; Fiedler, 1984; Middleboe and Johansen, 1991). Therefore, the present discussion will focus on new developments and some aspects that have received less attention.

a. The emission source The choices of glass type, tube shape, and sealed versus permanent cells, all of which are closely tied to methods used for sample preparation, are discussed in Section II.B. Excitation of emission has been reported using RFs from approximately 1 MHz to the standard microwave generator at 2450 MHz, with both fixed and variable power, usually in the 20–30-W range. Satisfactory operation has been reported over the whole frequency range, but lower excitation frequencies (up to about 150 MHz) generally result in less sample heating and a more stable discharge. Variable power is preferable because it allows the discharge to be run at the lowest power necessary to maintain a stable discharge. A medical electrosurgery unit has also been used as an RF source with variable power and can be conveniently controlled by a foot switch. With an electrosurgery unit, and with the discharge turned on only during the scan, there was little tube heating and no fan was needed (Preston *et al.*, 1981). However, more recent experience with a newer electrosurgery unit (Preston, unpublished) was unsatisfactory. Based on a theoretical analysis of the characteristics of the plasma discharge, a novel and inexpensive RF source has recently been developed (Hoult and Preston, 1992, *vide infra*).

The RF power is coupled to the gas through a microwave cavity or antenna (Fiedler and Proksch, 1975). In the Jasco instrument, the sample tube is held by two spring clips about 50 mm apart. With an electrosurgery unit (Preston *et al.*, 1981), a cavity was made by connecting the active and ground leads to two hollow aluminum cylinders that fit loosely over the sample tube. The gap between cylinders can be adjusted with a set screw on the top cylinder; 10–12 mm is suitable for most tubes, while reducing the separation to 2–5 mm will often produce a discharge in a tube that otherwise fails to start. Timperley and Priscu (1986) describe a cavity with matching controls used to maximize the emission intensity for samples of 1–110 μg total N.

It is also usually necessary to use a Tesla coil or built-in "snap starter" to apply a high voltage to the tube for 1 sec or 2 before applying the RF power. This can be done just before the sample is placed in the cavity or (where cavity geometry is suitable) while the sample is in the holder. Once initially excited, many tubes can be cycled on and off with short delays without further application of this "preexcitation." This effect may be due to build-up of static charge on the walls of the tube.

Despite the proliferation of RF sources, there has been little attempt to understand the properties of the discharge and, thus, to improve the source design. The RF source also remains the single most difficult barrier to those wishing to build an optical emission analyzer rather than use commercial instruments. Based on a theoretical analysis of the plasma discharge, Hoult and Preston (1992) have recently developed a compact inexpensive source based on a single transistor, 100-MHz, 1-W oscillator. The device uses impedance matching to mimimize the power requirements and a simple feedback circuit with a phototransistor to control the emission intensity. It is compact and easily mounted on an optical rail. Tube heating is minor, and discharge stability is such that a tube is normally left "on" when the system is not in use to maintain optimum stability of the entire system.

b. Optical components. The light emitted from the tube is focused on the entrance slit of the monochromator by a quartz lens (plano-convex or bi-convex) of aperture (F number, defined as diameter over focal length) similar to that of the monochromator. The lens formula describes, to a first approximation, the relationship between focal length (FL), distance from object to lens (*o*), and distance from lens to image (*i*):

$$1/\mathrm{FL} = 1/o + 1/i. \tag{1}$$

The equation can be solved with both *o* and *i* equal to 2(FL); focal lengths of 50–80 mm are suitable in this application. The lens should be mounted in a holder that can be translated in the two axes perpendicular to the light path for optimization of the signal from each sample.

Figure 3 shows the optical arrangement of a Czerney–Turner grating monochromator. In this configuration, the focusing elements are concave mirrors, whereas in monochromators with a concave grating the grating serves as both dispersing and focusing elements. Leicknam *et al.* (1968a,b) used a quartz prism monochromator, and the older Statron models (Meier and Müller, 1965) a sodium chloride one. The main advantage of grating over prism monochromators is the much higher throughput, leading to greater sensitivity. In addition, sodium chloride prisms require protection from moisture.

Several factors determine the performance of a monochromator. The FL for a monochromator is the distance between the entrance slit and the focusing element, whether a lens, mirror, or concave grating. The linear dispersion increases with increasing FL, as do cost, physical size and weight, and environmental requirements for vibration protection and temperature control. Linear dispersion measures the monochromator's ability to spread the spectrum across the exit slit and is usually expressed as reciprocal dispersion (RD), i.e., nanometers (of spectrum) per millimeter of exit plane. Aperture is the ratio of the focal length to the grating diameter; a lower F number indicates a wider collection angle and greater photon-gathering ability. Throughput is the overall effectiveness with which the monochromator transmits light and is much lower in prism monochromators. Stray light is radiation of wavelength other than that desired. Thus, high throughput is of little advantage if the level of stray light is also high.

For ^{15}N analysis, it is possible to use monochromators with RDs up to about 3.5 nm mm^{-1}, but an RD of <2 nm mm^{-1} is preferable. The monochromator should have a range of scanning speeds and either fully adjustable slits or sets of exchangeable ones. As mentioned in Section I.B, at higher resolution the rotational structure is resolved.

c. Exit system/detection/data processing Early work was carried out using spectrographs with photographic plates. In most systems currently used, the monochromator is scanned through the region, and the dispersed light passes through a single exit slit, is detected by a PM tube, is amplified, and is presented on a chart recorder. The PM tube, selected for high sensitivity in the near-UV region, should be positioned in a light-tight housing directly after the exit slit. Some systems include a chopper before the entrance slit to reduce detection of stray light and shot noise in the detector.

Some innovative detection and data-processing systems have been designed to attempt to overcome limitations inherent in the conventional scheme of scanning the monochromator and using a single detector. The scan speed is limited by the ability of components to respond, and small changes in emission intensity during the scan time are reflected in variations in the ratios of peak heights. Analysis systems have therefore been developed with dual or multiple exit slits for fast acquisition (Sections II.A.2 and II.A.3). More detailed discussion of detection and data-processing systems is beyond the scope of this chapter, and specific information should be obtained from the manufacturers or from the descriptions of individual systems reported in the literature. It should also be noted that the development of more complex systems has rarely resulted in a genuine improvement in overall precision and accuracy.

d. System mounting and housing Most commercial systems are built into a single housing including source, monochromator, electronics, and chart recorder. In the case of the Statron N0I-6e, the sample preparation line is also built into the same unit. When a system is assembled from components, it is more convenient to mount the items in the optical path (source, lens, chopper, monochromator, and detector) on a separate base. Other electronic components and sample preparation units for permanent discharge cells can be arranged for convenience in operation and maintenance and for safety. Placement of the RF power source away from the other electronic components reduces interference.

The optical components should be mounted on a heavy baseplate that is resistant to deformation and protected from vibration. In practice, the requirements for a small monochromator are easily met. A suitable baseplate can be made from aluminum 20–25 mm thick, with a grid of tapped holes for mounting components. The plate should be anodized or painted black. In most circumstances, sufficient vibration protection can be achieved using the inexpensive pads available for isolating balance-bases, or even four large rubber stoppers drilled and bolted to the plate. A short piece of optical track is suitable for mounting the source and lens and, in some cases, the monochromator as well. It is not necessary to purchase the highest quality of optical rail and mounting components, and suitable items may be available from disused atomic absorption or UV-VIS spectrometers.

Table II
Manufacturers and Distributors of Equipment for Optical Emission ^{15}N Analysis

Jasco N-150	Jasco International Co., Ltd. 2-4-21, Sennin-Cho, Hachioji City Tokyo 193, Japan Tel. 0426-66-1321
	Jasco Incorporated 314 Commerce Drive Easton, Maryland 21601 USA Tel. (301) 822-1220
Sopra GS 1	Sopra 68, rue Pierre Joigneaux F 92270 Bois-Colombes France Tel. (1)242.04.47 or (1) 781.09.49
Statron NOI-6e	(Manufacturer) Akademie der Wissenschaften der DDR Zentralinstitut fur Isotopen- und Strahlenforschung DDR-7050 Leipzig, Permoserstr. 15 Tel. 23920
	(Exporter) Isocommerz GmbH Binnen- und Aussenhandelsunternehmen für radioaktive und stabile Isotope Export Office DDR-7050 Leipzig, Permoserstr. 15 Tel. 2392327

The cover for the optical components should provide dust protection, exclude stray light during operation, and allow easy access for sample changing and maintenance. A metal cover (painted black inside) with a portion that can be opened is suitable. An inexpensive cover can also be made from light-tight fabric with a black lining over a frame made from components used to build laboratory racks.

e. Safety features As the discharge tubes emit UV radiation, eye protection is required. Care should be taken to avoid letting the Tesla coil discharge skip to metal or electronic components. The work space should be uncluttered, and electrical cords kept organized and off the work surface. Where a He-Ne laser is used for alignment, or a sodium or mercury lamp for testing or trouble-shooting, suitable eye protection must be used.

2. Commercial Instruments

a. Jasco Table II lists recently available information on instrument manufacturers. The Japanese Jasco N-150 analyzer uses an RF source at

13.56 MHz and 30 W, a Czerney–Turner monochromator, a PM tube detection, and a built-in recorder. The light source is chopped at 225 Hz, and scanning is carried out with a stepping motor and cam mechanism. A six-position sensitivity selector provides automatic changing of recorder amplification (from ×1 to ×32) for the $^{14}N^{15}N$ and $^{15}N_2$ peaks. Enrichments are determined using a calibration curve made using standards also available from the manufacturer. A sample preparation system is also available for both Rittenberg and Dumas procedures.

b. Sopra The French Sopra instrument (Guiraud and Fardeau, 1980) is based on a monochromator of 0.4 m FL with a concave grating and 1 nm mm^{-1} RD. The grating position is fixed, and scanning from 297 to 300 nm is achieved by rotation of a quartz plate. The RF source operates at 27 MHz and 20 W. An output is provided for a chart recorder, and as for the Jasco, automatic switching of sensitivity is provided. A preparation system is available for Dumas combustion using straight Pyrex tubes, and a set of small ovens are positioned over the tubes for degassing. Applications with the Sopra instrument are described by Martin *et al.* (1981) and Lemasson *et al.* (1982).

c. Statron The older N0I-5 and Isonitromat instruments, made by Statron in Leipzig (formerly East Germany), used a monochromator with a sodium chloride prism (Meier and Müller, 1965). The Isonitromat was an automatic system, incorporating Rittenberg sample preparation and a permanent discharge cell. These are no longer available and have been replaced by the N0I-6e (Meier and Mauersberger, 1982; Meier, 1986). This includes an integrated sample preparation unit using the Rittenberg procedure and permanent discharge tube or alternative use of sealed tubes. Components include an RF unit operating at 27.12 MHz and 50 W and a 0.5-m FL Ebert monochromator with RD 1.5 nm mm^{-1}. For rapid data acquisition, scanning is replaced by an oscillating exit slit, and data are collected in four spectral regions corresponding to background and the three N_2 bandheads. Operation is completely under microcomputer control. The disadvantage of this system is that operation is complex, and the chemical and optical systems are built into the same housing.

3. Other Systems

Leicknam *et al.* (1968b) modified a high-resolution quartz prism spectrograph by adding a scanning mechanism, exit slit, PM tube detector, and RF source operating at approximately 50 MHz. Preston *et al.* (1981) assembled a system using an electrosurgery unit, a chart recorder, and a modified Bausch and Lomb Spectronic 502 UV-VIS spectrometer. The RD of the monochromator was 1.6 nm mm^{-1}, but it was necessary to replace the large fixed slits, reducing their size to approximately 50 μm in width and 5 mm in height. The instrument was operated with manual control of scanning, amplifier, and chart recorder, and simultaneous control of the RF source by a foot switch. Enrichments were determined using a calibration curve, and precision and accuracy were comparable to that reported for the Jasco and several other instruments. Because the RF source was low frequency (around 1 MHz), and the excitation was switched off while the monochromator was being reset to the starting frequency, there was little heating of the tubes, and no fan was needed in the sample chamber.

A new version has been assembled with improved physical layout, allowing greater ease of data collection and manipulation. It is based on a J-Y Optical (Instruments SA Inc.) HR-320 0.32-m F4.2 Czerney–Turner monochromator with an RD of 2.4 nm·mm^{-1} and variable slits. A Spectra-Link interface (Instruments SA Inc.) controls scanning, PM tube voltage, and detection with a dual channel module, which uses either photon counting or direct current measurement depending on the signal level. Output can be directed to a chart recorder or stored in either of two 1000-point memory channels for single or multiple scans. A modern electrosurgery unit proved to be unsatisfactory as an RF source and has since been replaced by the novel plasma discharge previously described in Section II.A.1.a (Hoult and Preston, 1992). The source, lens, monochromator, and PM tube are mounted on an aluminum baseplate with a fabric cover and rubber feet (Section II.A.1.d). With a quartz focusing lens (76-mm FL), straight-walled Pyrex sample tubes, and both slits at 10 μm, resolution and sensitivity are adequate for enrichments down to natural abundance with one scan. However, routine analysis is usually carried out using time-averaging of two scans to enhance the signal-to-noise ratio of samples with low ^{15}N enrichments. The Spectra-Link system allows flexibility in operation, because the plotter parameters do not have to be preadjusted, and stored signals can be plotted with suitable scaling. For samples with low ^{15}N enrichment, the stored spectrum is plotted twice from memory at different scales.

Burridge and Hewitt (1980) used an Optica CF4 spectrometer modified to permit a greater range of scanning speeds. Their instrument had an RD of 1.9 nm mm^{-1}1 and 40-μm slits. Signals were detected by a PM tube and sent to a chart recorder via an autoranging digital voltmeter. The discharge tube was positioned about 15 cm in front of the entrance slit without a focusing lens. The permanent discharge tube was filled with ammonia, which decomposes to form N_2 in the discharge (Section III.B). The 2 → 0 bands were used for samples of natural abundance and greater, and the 1 → 0 bands for samples depleted in ^{15}N.

Eierman and Piepmeier (1986) used a high-resolution echelle monochromator with four PM tubes to monitor simultaneously two N_2 and two background signals. Nitrogen gas is excited in a glass cavity with a quartz window. To reduce memory effects in the discharge, a disposable glass liner (which does not cover the window) is changed after each nitrogen sample, and a series of He samples is excited before admission of the new He/N_2 sample. The system is interfaced to a computer for acquisition and data analysis. Memory effects, limiting sensitivity, plasma stability, and data analysis were investigated using standard gas samples, but sensitivity and precision in this complex prototype were not in fact improved beyond those generally obtained.

Lipschultz (1984) and Lipschultz *et al.* (1985) used a 0.5-m-FL monochromator with dual slits at 297.68 and 298.32 nm. A chopper operating at 300 Hz alternates imaging of each signal onto a PM tube. The resultant signal is passed through gating electronics synchronized to the chopper, and the intensity for each bandhead is measured by photon counting. A microcomputer reads the counters, stores the data, and computes the isotopic ratio. Improvement of precision to ±0.002 atom% ^{15}N was attributed (Lipschultz, 1984) to photon counting detection, high-resolution monochromator for complete separation of the isotopic bandheads, and elimination of scanning. Other sources of error were found to be variations in the RF power and N_2 gas pressure and fluctuations in the coupling of the RF energy to the plasma. Vycor or quartz tubes were also recommended for highest precision.

Timperley and Priscu (1986) combined an atomic absorption spectrometer with an inexpensive RF generator (maximum output 20 W at 27.5 MHz) and the cavity described in Section II.A.1.a. The latter is placed at the position normally occupied by the light source. This arrangement allows the atomic absorption spectrometer to be used for both purposes, with very low cost for the added ^{15}N analysis capability.

Two automated systems for ^{15}N analysis have been described. Therion *et al.* (1986) combined the analysis of total N and ^{15}N by coupling the output from a Carlo Erba Model NA1500 automatic nitrogen analyzer to the optical emission spectrometer. The He-N_2 output from the analyzer is excited in a discharge cavity by a microwave generator at 2450 MHz. The lines are resolved by a Jarrell-Ash 0.25-m-FL monochromator with an RD of 1.65 nm mm^{-1} and 50-μm slits. Rapid scanning is achieved by a rotating quartz plate positioned behind the entrance slit. Three intensity measurements are made, two for the $^{14}N_2$ and $^{14}N^{15}N$ peaks and one at a spectral minimum. To compensate for fluctuations in the source, the intensities of the analytical lines are constantly related to the output from a second PM tube that measures the intensity of the 337.1-nm bandhead directly through a narrow-pass interference filter. The measurement cycle and data analysis are under computer control. Standard deviations were reported as better than ±0.02 atom% ^{15}N for biological samples and lower for standards. No significant memory effect was found in the discharge cell.

In the instrument described by Goulden and Salter (1979) and Salter (1981), ammonium chloride samples are injected into a stream of purified helium and pass into a reactor tube held at 590°C. Reaction with soda lime in the first part of the tube generates NH_3, which is decomposed to N_2 and H_2 by a platinum–rhodium catalyst. N_2 is separated from H_2 and water on a molecular sieve column and passes through a quartz tube in a microwave cavity. A Rank-Hilger D331 double monochromator with RD 1.3 nm·mm^{-1} was modified for dual-wavelength measurement to one photomultiplier and a reference measurement to the second. The system supplies direct printout of ^{15}N enrichments. The uncertainty for low-enrichment, biological samples is reported as ±0.01 atom% ^{15}N, with better performance for standards. Memory effects in the discharge cell were insignificant.

B. For Sample Preparation

This chapter will focus on the technique most usually associated with optical emission analysis, i.e., sealed tubes and Dumas sample conversion. The components required are a vacuum line and muffle furnace. Fiedler and Proksch (1975) have discussed vacuum systems for ^{15}N analysis, so the treatment here will be brief. Figure 4 shows the basic components of a vacuum system for Dumas preparation of sealed samples. They are a two-stage rotary pump, a diffusion pump with bypass, a gauge, and a glass manifold (Fig. 4a). The glass manifold may include a portion for admitting argon or other inert gas to the system (Fig. 4b). As discussed later, many Dumas sample preparation procedures involve rotation of the manifold, or part of it, and this is most easily accomplished using ground-glass joints. Vacuum components are available from many commercial sources, and information should be obtained from the individual manufacturers. In general, connections should be kept short, and too many joints and small apertures avoided. Teflon stopcocks are recommended. The sample tubes may be joined to the outlets using metal tubing connectors with O-rings, such as Cajun Ultra-Torr fittings. These are far preferable to the outmoded wax or rubber tubing connections still described as standard in the literature.

Mercury diffusion pumps should be avoided for safety reasons, and also because mercury can spread through the system if the cold trap or water cooling fails during operation. A metal diffusion pump with oil of low

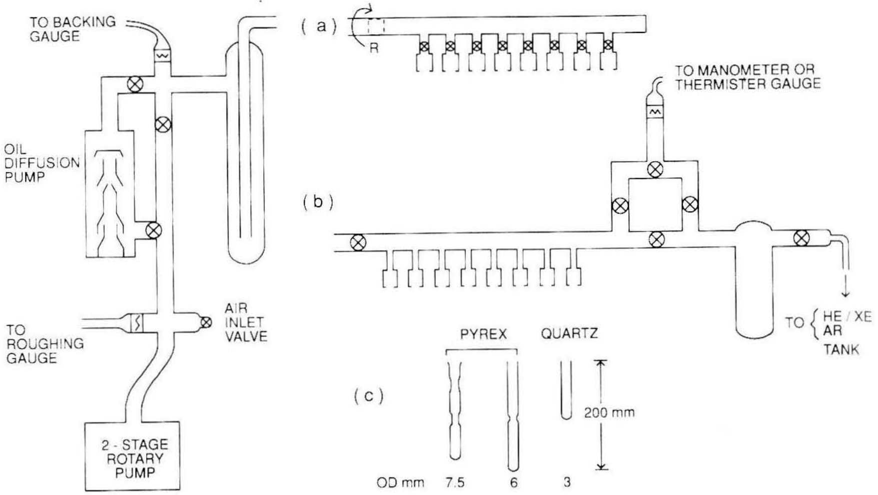

Figure 4 Vacuum system for Dumas preparation of sealed tubes with manifolds with optional rotatable joint (a) and for admission of inert gases (b), and Pyrex and quartz designs of sample tubes (c).

vapor pressure is preferable for safety and effectiveness. The diffusion pump should have a bypass, so that it is never exposed to high pressure while hot. However, with modern diffusion pump oil and careful operation, a liquid nitrogen cold trap is not necessary. A convenient diffusion pump–bypass combination is made by Edwards High Vacuum. Glass and metal components may be connected using metal-glass seals, short pieces of rubber tubing, epoxy glue, or, preferably, components for quick-coupling of glass and metal tubing of specific diameters. The latter are available, for example, from Edwards High Vacuum and allow quick separation and reassembly of components as well as flexibility in design of glass–metal systems.

There are several types of vacuum gauges, each applicable to a certain pressure range. Because the technology is constantly developing, the manufacturers should be consulted for information on suitable gauges and combinations. For most sample preparation procedures, a Penning gauge (10^{-3}–10^{-8} kPa) plus a Pirani or thermocouple gauge (1–10^{-5} kPa) is satisfactory. It should be noted that most types of gauges are calibrated for dry air or N_2, and correction curves are needed for other gases, such as argon; these are usually supplied with the gauge. Several different units have been used to report pressure (Table III), but the correct SI unit is the Pascal or kilopascal (Pa, kPa). In some publications, atm and mbar may be

Table III
Conversion Factors for Pressure Units. Correct SI Units Are Pa and kPa.

	1 Pa	1 kPa	1 mbar	1 atm	1 torr	1 mm Hg
1 Pa	1	10^{-3}	10^{-2}	0.987×10^{-6}	7.5×10^{-3}	7.5×10^{-3}
1 kPa	10^{3}	1	10	0.987×10^{-3}	7.5	7.5
1 mbar	10^{2}	10^{-1}	1	0.987×10^{-3}	7.5×10^{-1}	7.5×10^{-1}
1 atm	1.013×10^{5}	1.013×10^{2}	1.013×10^{3}	1	7.6×10^{2}	7.6×10^{2}
1 torr	1.333×10^{2}	1.333×10^{-1}	1.333	1.316×10^{-3}	1	1
1 mm Hg	1.333×10^{2}	1.333×10^{-1}	1.333	1.316×10^{-3}	1	1

acceptable but torr and mm Hg should not be used. With an E2M5 two-stage roughing pump and Diffstack diffusion pump from Edwards High Vacuum, a glass manifold, glass–teflon stopcocks, and Cajon Ultra-torr fittings, we quickly and routinely achieve a vacuum of better than 1×10^{-5} Pa (10^{-7} torr).

There are considerable advantages in building rather than purchasing a sample preparation system for ^{15}N analysis. Components are widely available, and a system can usually be designed to suit individual requirements at lower cost, with higher quality components and better local access to service and parts.

Safety features should be part of the sample preparation system. The area should be well ventilated, uncluttered, and away from walls or flammable materials due to operation of the oxygen–gas torch. Diffusion pumps should have thermal cutout switches, in case the cooling water fails, and be mounted on a heat-resistant surface. The vacuum system should be free of protruding components that can be easily broken. If glass gas reservoirs are used, they should be kept small (0.5–1.5 liters) and wrapped with plastic electrical tape in case of breakage. Cooling water connections should be tight and checked regularly to avoid floods. Electrical connections should be as short as possible, tidy, and kept away from possible contact with water or hot glass. The bench surface should be covered with heat-resistant material. Gloves and tongs should be available for handling hot glass or reagents. Gas tanks should be secured to good supports, and all connections kept tight. Eye protection (didymium–glass goggles) must always be used when glassblowing to avoid excessive exposure to the yellow emission of sodium.

For the N_2 discharge, both sealed tubes and flow-through or refillable cells have been used. The material must have sufficient transparency at the working frequency. In addition, it must be easy to work and able to withstand high temperatures for degassing and carrying out the Dumas reaction. Quartz is completely transparent at 300 nm; it has an annealing point of 1100°C and softening point of 1700°C. Vycor glass has a softening point of 1530°C and also a high transmittance at 300 nm, but its cost is also similar to that of quartz. Working with quartz and Vycor requires a hydrogen–oxygen flame. Pyrex, which softens at 570°C, is easily worked with an oxygen–gas flame, and has a transmittance of approximately 10% for a 10-mm thickness at 300 nm (Müller, 1973). To increase the signal available from Pyrex tubes, they have conventionally been made with a thin-walled constricted center portion (Fig. 4c); this, however, is completely unnecessary.

Several reports have been made of the effects of Pyrex tubes on isotope-ratio determination (Keeney and Tedesco, 1973; Lloyd-Jones *et al.*, 1975; Lipschultz, 1984). Because the transmittance of Pyrex declines rapidly below 300 nm (Müller, 1973), the peak ratios will be influenced by small variations in wall thickness and differences among batches of Pyrex. These effects will be exacerbated by intensity fluctuations in the intense discharge generated in the thin-walled section, especially with high-frequency excitation. Completely satisfactory results can be achieved, however, with straight Pyrex tubes of 1-mm wall thickness (Martin *et al.*, 1981; Preston *et al.*, 1981; Lemasson *et al.*, 1982; Ta and Joy, 1986; Timperley and Priscu, 1986), which can be inexpensively made in the laboratory (Fig. 4c). The slight constriction is an aid for sealing but is not essential.

The sample size is determined by the requirement for a final gas pressure in the range of 0.3–0.7 kPa in the discharge tube (Broida and Chapman, 1958). This can be calculated using the gas law:

$$n = PV/RT \qquad (2)$$

where n is the number of moles of N_2 gas, P the pressure in Pa, V the volume in cubic meters, T the temperature in kelvins, and R the universal gas constant, which in SI units is 8.314 J $(\text{K mol})^{-1}$. For example, a final pressure of 0.5 kPa in a tube of 1 ml at 25°C requires 0.202 μmol N_2, or 5.65 μg N $[(500 \times 1 \times 10^{-6})/(8.314 \times 298)]$. Dumas preparation in sealed tubes usually requires 5–20 μg of total N. Larger amounts of up to 200 μg of total N may be used in Rittenberg procedures or other methods in which the tube or cell is filled after conversion to N_2.

Samples as small as 0.2 μg total N can be analyzed by using a partial pressure of a noble gas to sustain the discharge (Cook *et al.*, 1967; Goleb and Middelboe, 1968; Fiedler and Proksch, 1975). Martin *et al.* (1981) to analyzed samples of 0.5 μg N using tubes with 0.3 ml internal volume, while Timperley and Priscu (1986) analyzed tubes with 1–110 μg N using a cavity with matching controls. The automatic systems described previously (Goulden and Salter, 1979; Salter, 1981; Therion *et al.*, 1986) use He/N_2 mixtures in flow-through cells and require only 2–20 μg total N. An admixture of He generates a very bright, but short-lived, discharge, whereas with He-Xe mixtures or Ar the discharge is less intense but much longer-lived. Another approach is to use isotope dilution, in which a known amount of natural-abundance N is added to the sample (Fiedler, 1984; Middleboe and Johansen, 1991).

III. PROCEDURES

A. For Spectroscopic Analysis

For commercial instruments, the manufacturers' instructions should be followed. The instrument must be allowed to warm up and should be protected from large temperature fluctuations during operation. Unless a high-voltage "trigger" starter is built in, it is necessary to preexcite tubes with a Tesla coil (Section II.A.1.a). The PM tube should never be exposed to room light while at high voltage. For other systems, operation will vary, but the comments just made also apply. Detailed information can be found in the literature and, for individual components, in the manufacturers' instructions. It may not be commonly known that PM tubes require approximately 24 hr of operation to reach stability; we now leave the system on and monitor the emission signal from a tube when not in use.

A few comments will be made here on the initial setup and troubleshooting of the optical components in noncommercial systems. The best operation requires maximum gathering of signal light while minimizing stray light. Therefore, the cover should be light-tight, and interior surfaces black and nonreflective. Initial alignment of optical components can be carried out using a low-power He-Ne laser at 632.8 nm in a darkened room. The monochromator is set at 632.8 nm, the PM tube and housing removed, and the laser shone in through the exit slit, out the entrance slit, and through the lens and discharge tube. With the monochromator cover off, the laser beam should also fall in the center of the mirrors and grating. A laser can also be used to measure slit widths from the spacings of the diffraction pattern produced by passage of the laser through the slit (the formulae are available in basic optics or physics texts). Appropriate goggles must be used for eye protection during laser operation.

It may occasionally be necessary to adjust the alignment of the slits or to check the resolution independently of that found for the N_2 lines. In general, final alignment of the monochromator components is done by the manufacturer, and adjustment should only be attempted if absolutely necessary. The resolution can be conveniently checked using the mercury lines at 365.0 and 365.5 nm or the sodium lines at 589.0 and 589.6 nm. If adjustment is necessary, it should be done in extremely small increments, and the direction of changes noted. Also, a scan should be recorded initially for subsequent comparison. With the monochromator cover off, and in a darkened room, the image of the slit on the grating and mirrors should be central and parallel to the grooves. Appropriate eye protection must be used with these light sources—glassblowing goggles for the sodium lamp and UV goggles for mercury.

In setting up individual sample tubes for analysis, it is worth monitoring the signal at some frequency in the analysis region and adjusting the position of the lens to maximize the signal. If the lens position has accidentally been overadjusted, the image of the discharge tube can be easily visualized on a piece of white tissue paper with the room darkened, and the direction of corrections followed.

B. For Sample Preparation

In general, procedures leading to optical emission analysis tend to be miniaturized compared to those used for mass spectrometry, and diffusion is often used to concentrate ammonia, but any procedure resulting in ammonium chloride or sulfate may be used as a preliminary stage. Both the Rittenberg and Dumas procedures are used to generate N_2 gas from ammonium salts. In addition, optical emission analysis is particularly suitable for application of the Dumas procedure to intact samples. Methods devised to separate forms of nitrogen, and applications in specific areas, are described in other chapters. Sample preparation has been well documented elsewhere (Fiedler and Proksch, 1975; Hauck, 1982; Fiedler, 1984), so only selected Dumas procedures and new developments will be discussed here.

The conventional Dumas procedure for optical emission is carried out using tubes with a central constriction, a tipping vacuum line, and heating of the CaO and CuO within the tubes under vacuum. As described by Fiedler and Proksch (1975), the sample (in ammonium form) is dried in a small quartz or glass capillary tube. This is put into the upper part of a discharge tube that has been precleaned, usually by heating at 550°C. Quartz discharge tubes are straight-sided and about 3 mm in diameter, whereas Pyrex tubes have usually been made with a constricted central portion (Fig. 4c). The CaO, which is often briquetted with Al_2O_3 for convenience in handling (Fiedler and Proksch, 1975), should be preheated to 1000°C in a muffle furnace to decompose carbonates. The CuO is usually in wire form and should also be preheated at 650°C, although in some procedures this heating occurs only under vacuum during preparation of the discharge tube. The CuO and CaO are placed above the sample capillary. Where nitrates are present, a few grains or wires of elemental Cu are also added.

The discharge tubes are fastened to the vacuum line in a horizontal position and evacuated. The empty lower portion is degassed by heating with a hand torch, the vacuum line is tipped about 30° from horizontal, and the sample capillary is then moved (by gentle tapping) to the bottom of the discharge tube. To degas the reagents, the lower portion of the tube is then heated with a hand torch. Additional degassing may be carried out by applying a Tesla coil discharge to the tubes. The completion of the degassing procedure can be ascertained by following the vacuum gauge. When degassing is complete, the tubes are rotated to the vertical position and sealed. For very small samples, inadequate to generate a sufficient final gas pressure, a noble gas such as He, Ar, or a He-Xe mixture is added before the tubes are sealed. The exact pressure that should be admitted can be determined using a series of test samples. The tubes are baked for several hours, at approximately 550°C for Pyrex and 800°C for quartz or Vycor. The required oven temperatures can be calibrated using blank sealed tubes. After baking, tubes should be left for a few hours before use; this seems to be necessary to allow absorption of impurity gases by the CaO.

Although many variations have been reported, the basic procedure is adequately covered in Fiedler and Proksch (1975). Many variations of Dumas preparation procedures may be found in the literature (see References), and only a few points will be noted here. Some of the modifications reported arise from the self-imposed challenges of working around the central constriction of Pyrex tubes and applying the correct level of heating to different components; these problems disappear with the use of straight-walled tubes and preheating of reagents in a muffle furnace.

Several authors find the absorption of impurity gases by CaO to be incomplete or consider that the CaO itself contributes impurities. Therefore, Lemasson *et al.* (1982) and Leicknam *et al.* (1968a) operated the discharge with the lower end immersed in liquid nitrogen. The author has recently adopted a procedure that eliminates the CaO completely. After baking, the end of the tube is immersed in liquid nitrogen, cut off, and discarded. Many of the concerns surrounding degassing, generation of impurity gases, and incomplete combustion seem to be associated with fairly large samples and tubes and also heating of the reagents only in the discharge tube, where the upper temperature is limited. There are also many discussions of memory effects in reused quartz tubes. If disposable Pyrex tubes are used, this is not a problem, and recent studies of memory effects in permanent discharge tubes have usually found that discharging several times with He or natural-abundance N_2 reduces memory effects to a negligible level.

Simpler Dumas procedures have been described (Martin *et al.*, 1981; Preston *et al.*, 1981; Lemasson *et al.*, 1982; Ta and Joy, 1986; Timperley and Priscu, 1986). A generalized procedure follows, although the preceding sources should be consulted for details. The discharge tubes are straight-walled Pyrex, with an optional slight constriction to aid in sealing (Fig. 4c). The tubes are precleaned by heating at 550°C overnight and stored in a desiccator or in an oven at 90°C, protected from dust. The sample is placed in the bottom of the discharge tubes, usually within a capillary. However, the capillary is unnecessary; Preston *et al.* (1981) introduced samples directly to the discharge tube. Solids are shaken down by bouncing the tube, and solutions are introduced (50–100 μl) with a drawn-out Pasteur pipette and dried at 90°C. Tubes with samples may also be stored at 90°C or in a desiccator. CuO (wire or powder) and CaO are preheated at 650 and 1000°C, respectively, in a muffle furnace and may be stored in a vacuum desiccator for up to 2 days. There is no real need to briquette the CaO, and the presence of some white powder on the interior walls does not seem to degrade spectral quality. Cuprox, a copper oxide containing platinum catalyst on alumina (Coleman Reagent), is sometimes used in place of CuO.

In this simplified procedure, the preheated reagents are added to the tubes (CuO and Cu first, then CaO), which are quickly connected in a vertical position to the vacuum line. The tubes are evacuated and then degassed by heating with a hand torch or small portable furnaces, with care to avoid excessive heating of the bottom of the tubes with the samples. A noble gas can then be added, if necessary for small samples. The tubes are sealed off and heated at 550°C in a muffle furnace for a few hours or overnight. An aluminum block with vertical holes for tubes and side grooves for gripping with tongs makes a convenient sample holder.

Once tubes have been prepared, problems may be encountered with the discharge. Tubes that light briefly and then go out usually have insufficient gas pressure, especially if the discharge is reddish. Excess CO_2 will produce a bluish discharge of CO^+, which completely masks the N_2 emission at 297 nm. If tubes will not light at all, the pressure is usually too high; however, especially with intact samples, rebaking may help if the original combustion was incomplete. The RF power can be increased, and in the case of the cylindrical electrodes, the separation can be reduced to 2–3 mm. If there appears to be excess CO_2 or water, the bottom of the tube can be immersed in liquid N_2, sealed off, and discarded. A common cause of failure is glass leaks; either the bottom of the tube is originally made with a pinhole or the top is sealed with a very weak joint, so that any stress, including the discharge from the Tesla coil, causes a leak. Another common cause of failure with intact samples is too large a sample; in general, a set of test samples should be prepared so that the operator becomes accustomed to estimating the amount.

The amounts of reagents are not critical, but it seems better to use a generous amount of CaO—i.e., an amount sufficient to fill a 6-mm-OD tube to 10–12 mm. With CuO wire, two to three pieces about 5 mm in length are sufficient, or a "pinch" (the end of a microspatula) of powder. Where Cu is used, the amount is very small—less than one-tenth the amount of CuO used. Even though Cu is only required where nitrates are present, it often seems to be beneficial for intact plant and soil samples. With careful control of sample size, the success rate for pure chemical standards or ammonium samples should be close to 100%. With intact samples, where sample sizes are usually 1–10 mg, the success rate should still be 80–90%.

At least an estimate of N content is needed to use the Dumas procedure with sealed tubes. Total N may be determined as part of Kjeldahl procedures to produce samples in ammonium form. Where intact samples are used, total N must be determined separately, and an automatic combustion-type analyzer offers a quick alternative to Kjeldahl determination. As described in Section II.A.3, Therion *et al.* (1986) combined total N and ^{15}N analysis by coupling an automatic analyzer and an optical emission system. In the system used for preparation of discharge tubes by Ohmori *et al.* (1981), the N_2 gas produced is introduced into a McLeod gauge for measurement of the total quantity, and then an appropriate amount is collected in a discharge tube containing a molecular sieve.

Burridge and Hewitt (1980) used a system for filling a permanent discharge tube with ammonia, which decomposes to N_2 during excitation at 2450 MHz. This was devised to avoid the final step of conversion to N_2 and to be convenient for larger samples (1–30 mg total N). Unfortunately, other impurity gases are also produced, and the lower part of the discharge tube must be cooled to $-60°C$ to reduce interference from OH emission. The procedure seems to be no less complex than generating N_2 chemically, and large ammonium samples can be easily and accurately subsampled as solutions. Yamamuro (1981) claims improved accuracy with sealed tubes filled with ammonia, although each tube requires excitation at 27 MHz for several hours to complete the conversion of NH_3 to N_2; this hardly seems a generally applicable approach.

C. For Data Analysis

The equations relating ratios of peak heights to ^{15}N abundance have been discussed in Chapter 2 (Section III.D.4) on mass spectrometry. Optical emission has the advantage that, however the N_2 gas is produced, the distribution of isotopes is randomized in the discharge, so that only the two-peak formulae (see Chapter 2) need to be used. Procedures for spectral analysis have been discussed by Fiedler and Proksch (1975). Briefly, the isotopic lines are not completely resolved in most instruments, so that for samples of low enrichment, the $^{14}N^{15}N$ peak appears as a shoulder on the side of the much larger $^{14}N_2$ peak. In addition, the intensity distribution can be distorted by traces of impurity gases, especially CO_2, which generates a CO^+ band at 298.3 nm (Meier and Müller, 1965; Leicknam *et al.*, 1968a; Fiedler and Proksch, 1975). For these reasons, it is difficult to calculate ^{15}N abundances directly from ratios of peak heights.

Figure 5 shows spectra at low ^{15}N abundance, with horizontal baselines used to assess peak heights. Some users recommend drawing the baseline of the $^{14}N^{15}N$ peak by interpolating the intensity of the larger $^{14}N_2$ peak, as shown, for example, in Timperley and Priscu (1986). The latter procedure has been recommended for monochromators of 2 nm mm^{-1} RD or better (Fiedler and Proksch, 1975, and references therein), followed by direct calculation of atom% ^{15}N using the equations in Chapter 2 (Section III.D.4). In fact, it is almost always necessary to apply a correction based on a calibration with standard samples. It seems preferable to use horizontal baselines, which are less subject to human influence, and calculate results directly using a calibration curve of ^{15}N abundance versus R(28/29) or R(29/30), taking into account, of course, changes in sensitivity scale. Preston *et al.* (1981) found good agreement between the standards supplied by Jasco and those made by diluting solutions at 90 or 99% ^{15}N abundance with those of natural abundance. The calibration was linear up to about 3% ^{15}N abundance; higher values were read off the curve, although fitting routines can be used for nonlinear calibrations. The 28/29 ratio was used for up to 30–40% ^{15}N, and the 29/30 ratio above that.

Figure 6 shows the calibration for low ^{15}N abundance for the instrument

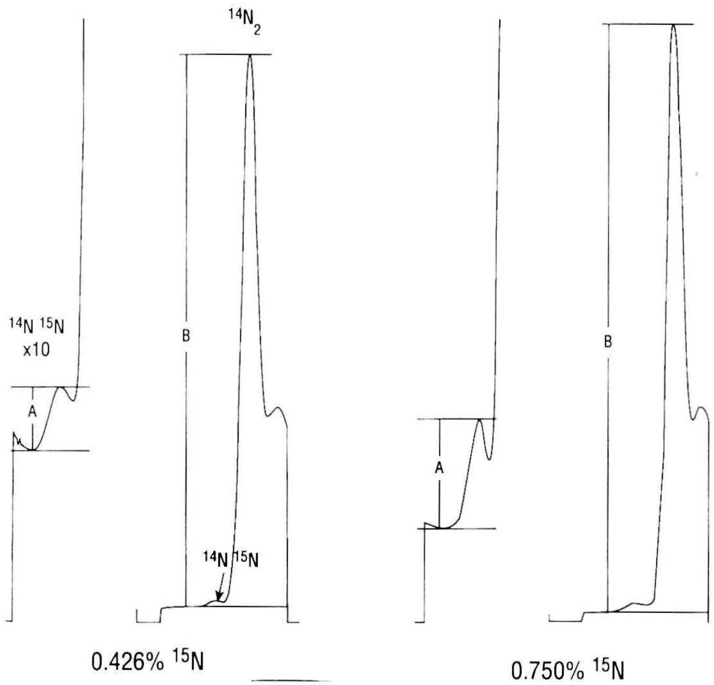

Figure 5 Emission spectra for samples at low levels of ^{15}N abundance, showing horizontal baselines used to measure peak ratios. Spectra were obtained using the new system described in Section II.A.3. [Reproduced, with permission, from Hoult, D. I., and Preston, C. M. (1992). An inexpensive plasma discharge source for molecular emission spectroscopy with application to ^{15}N analysis. *Rev. Sci. Instrum.* 63, 1927–1931.]

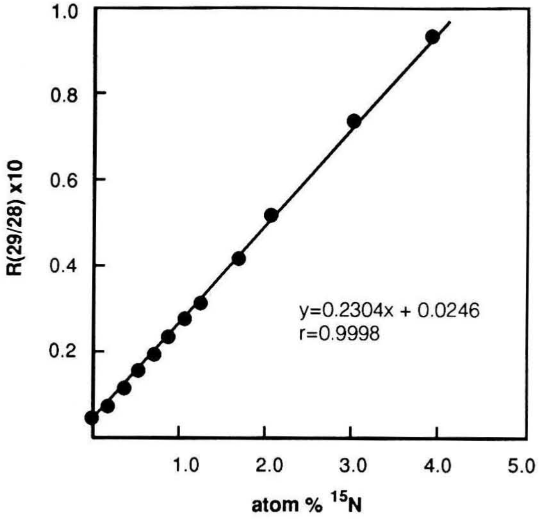

Figure 6 Calibration for low ^{15}N abundance for the new system described in Section II.A.3.

described in Secton II.A.3. Standards were prepared by mixing $(NH_4)_2SO_4$ at 99% ^{15}N and natural abundance, and the calculated values were confirmed by analysis with a VG Sira 9 mass spectrometer. Samples below natural abundance were prepared using ^{15}N-depleted $(NH_4)_2SO_4$ supplied by ICON.

Rather similar levels of precision and accuracy have been reported for many diverse instruments and procedures. For routine analysis of biological samples at natural abundance, precision and accuracy are both usually ±0.01–0.02 atom% ^{15}N, or a percent error of ±(2.7–5.5) (e.g., Fiedler and Proksch, 1975; Craswell and Eskew, 1991; Hoult and Preston, 1992). This allows measurement of enrichments down to about 0.02% above natural abundance. Results tend to be a little better at higher enrichments or with pure standards. Lipschultz (1984) and Yamamuro (1981) reported improved error limits as low as ±0.002 atom% ^{15}N, or 0.5% error.

Concern is often raised about the sampling error likely to occur with the very small sample sizes used in the direct Dumas method, but, in practice, there appears to be little difference between results found using direct conversion or preconversion to ammonium (e.g., Kristiansen and Paasche, 1982; this has also been the experience of the author). This sampling error would undoubtedly be a factor in achieving the highest precision. Even at the lowest reported error limits, optical emission analysis cannot be used to measure variations in natural abundance but is completely satisfactory for most biological investigations with ^{15}N enrichment. Sources of error were examined by Timperley and Priscu (1986), who concluded that the largest contribution arises from sample preparation, rather than from instrument performance. Martin *et al.* (1981) offer suggestions for minimizing this source of error, including precleaning of tubes and protection of samples, glassware, and reagents from external N contamination, especially ammonia.

IV. COMMENTS

Optical emission spectroscopy offers a simpler, less expensive method for ^{15}N analysis than mass spectrometry. It is also more suitable for very small samples, from 1–10 μg, although improvements continue to be made in this regard for mass spectrometry. Optical emission analysis is also well suited to Dumas sample preparation, from either intact samples or ammonium form. Problems arising from incomplete combustion or generation of impurity gases can be reduced to manageable levels with the very small sample sizes used for optical emission.

At least three sources of commercial equipment are available for optical emission, and all three offer sample preparation units. Many systems have also been described in the literature. A spectrometer system can also be assembled from commercially available components, often with advantages of lower cost, higher-quality components, greater flexibility, and simpler maintenance and upgrading. For sample preparation, it is almost always preferable to build a system tailored to the specific application.

The optical emission method was developed as a simple, cheap alternative to mass spectrometry. However, many developments have been reported that seem unduly complicated or expensive, without, in most cases, any significant improvement in error limits. This is particularly inappropriate when benchtop mass spectrometer systems are becoming less costly. For many small laboratories, in which ^{15}N analysis is not the primary activity, optimizing throughput at the final stage is not a priority, and considerations of low cost, simplicity, training requirements, flexibility, and integration with other analytical steps are more important.

Many diverse applications of optical emission analysis have been reported. Because these are covered in other chapters in this volume, the following brief summary highlights reports in which techniques for optical emission were featured. Studies of amino acids are described by Kano *et al.* (1975) with a modification (Ito *et al.*, 1976) to purify the silica gel by heating and washing, by Hirschberg *et al.* (1987) and Ta and Joy (1986), and plant/soil applications by Kanazawa and Yoneyama (1976), Lloyd-Jones *et al.* (1974, 1977), Macklon *et al.* (1990), Muhammed and Kumazawa (1976), Nannipieri *et al.* (1985; 1990), and Preston (1982). Studies in limnology and oceanography are found in works by Axler and Reuter (1986), Blackburn (1979), Goeyens *et al.* (1985), Kristiansen and Paasche (1982), Lemasson and Pages (1983), Lemasson *et al.* (1982), Lipschultz *et al.* (1985), Murphy (1980), Paasche and Kristiansen (1982), and Priscu and Downes (1985). Nitrogen fixation was studied by Hudd *et al.* (1980), MacRae (1975), and Lethbridge and Davidson (1983). A general bibliography of ^{15}N in nitrogen fixation is given by Faust (1986). Studies of denitrification have been made by Madsen (1977), Kjeldby *et al.* (1987), and Lippold (1984).

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4

Natural Abundance of ^{15}N: Fractional Contribution of Two Sources to a Common Sink and Use of Isotope Discrimination

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I. INTRODUCTION

Variation in ^{15}N abundance of various components of the biosphere is typically within 1 or 2% of that of atmospheric N_2 (0.3663 atom% ^{15}N; Junk and Svec, 1958). This variation results from isotopic discrimination. Though small, differences in ^{15}N abundance can be measured precisely, given care in sample preparation and suitable instrumentation. Such differences have been used in two ways: (i) to estimate the relative contribution of two *immediate* sources of N to a common sink (to be referred to as source identification), and (ii) to draw inferences concerning the mechanism or operation of the mechanism of a metabolic process by studying the isotopic discrimination associated with it.

A. Definition of the Unit for Expressing Natural ^{15}N Abundance

The unit used to express ^{15}N abundance at the naturally occurring level is δ^{15}N, the per mil ^{15}N excess (‰ ^{15}N).

$$\delta^{15}\mathrm{N} = \frac{R(\mathrm{spl}) - R(\mathrm{std})}{R(\mathrm{std})} 1000‰ \quad (1)$$

where the subscripts spl and std refer to sample and standard, respectively. The standard to which δ^{15}N is usually referred is atmospheric N_2. R has been defined as

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$$R = \frac{{}^{15}\mathrm{N}}{{}^{15}\mathrm{N} + {}^{14}\mathrm{N}} \quad \text{or} \quad R = \frac{{}^{15}\mathrm{N}}{{}^{14}\mathrm{N}}$$

Derivations often differ in their complexity depending on which definition of R is used. The former definition is generally more useful when the subject is source identification, the latter when the subject is isotope discrimination. At the level of natural abundance, these two definitions are operationally indistinguishable, because the difference in δ^{15}N values calculated using the two definitions is much smaller than the error of the measurement (see Appendix).

B. Isotope Discrimination: Background Information

Interpretation of data on the natural abundance of ^{15}N requires an understanding of the effect of isotope discrimination in altering the ^{15}N abundance of constituents of the N cycle.

1. Equilibrium isotope effects

Equilibrium constants are determined by differences in structure and energy of two species at equilibrium, and these differences are affected by isotopic composition (Bigeleisen, 1965). For example, in the equilibrium $H^+ + NH_3 \leftrightharpoons NH_4^+$, the equilibrium isotope effect causes ^{15}N to be approximately 2% (20‰) less abundant in NH_3 than in NH_4^+ (Hermes *et al.*, 1985). This equilibrium isotope effect can make a considerable contribution to variation in ^{15}N abundance in components of the N cycle. Volatilization of NH_3 from terrestrial and aquatic ecosystems enriches the residual NH_4^+ in ^{15}N, and this process may contribute substantially to the general (although not universal) elevation of ^{15}N in soils compared to atmospheric N_2.

2. Kinetic isotope effects

Differences in the rate of reaction of molecules bearing ^{15}N and ^{14}N result in differences in ^{15}N abundance between substrate and product.

a. *Intrinsic isotope effects* Each step in a reaction sequence has a characteristic (intrinsic) kinetic isotope effect, defined as $k_i/{}^*k_i$, where k_i is the rate constant for the reaction of molecules bearing ^{14}N in step i, and ${}^*k_i$ is the rate constant for the reaction of molecules bearing ^{15}N in step i. If the bonding to N is affected in step i, k_i will be different from ${}^*k_i$; i.e., the intrinsic isotope effect will be different from 1. Intrinsic isotope effects are usually, but not always, greater than 1 (a normal isotope effect) (Fry, 1970). Bond-breaking steps are universally associated with a normal isotope effect. Occasionally, the isotope effect associated with bond-making steps is inverse; i.e., in these cases, the intrinsic isotope effect is less than 1. The magnitude of intrinsic isotope effects decreases with increasing temperature, but in the range of physiological conditions this variation is minimal, and intrinsic isotope effects may be considered to be essentially constant.

b. The overall observed isotope effect for the entire reaction (β_{obs}) β_{obs} is defined as the ratio of the time rate of change in the concentrations of ^{14}N- and ^{15}N-bearing molecules normalized for their relative concentrations. This may be expressed as

$$\beta_{\mathrm{obs}} = \frac{\dfrac{d({}^{14}\mathrm{N})}{dt} \Big/ ({}^{14}\mathrm{N})}{\dfrac{d({}^{15}\mathrm{N})}{dt} \Big/ ({}^{15}\mathrm{N})} \tag{2}$$

The magnitude of β_{obs} is determined by the magnitude of the intrinsic isotope effects for the individual steps, their relative rates, and the mechanism of the reaction.

Biologically mediated reaction sequences generally consist of the following steps: (i) diffusion of substrate to the cell, (ii) transport of substrate into the cell, (iii) association of substrate with the enzyme, (iv) catalysis in one or more steps, and (v) release of product from the enzyme. The product of one enzymatic reaction is often the substrate for another. On occasion, more than one enzymatic reaction may compete for the same substrate. The result is a network of reactions, including major biochemical pathways. In the case of reactions of NH_3, the equilibrium between NH_3 and NH_4^+ may precede step (i). The isotope effect associated with diffusion of solutes through aqueous media is essentially 1.000 (Shearer and Kohl, 1986). Likewise, no measurable isotopic discrimination is associated with transport across membranes or with enzyme substrate association, unless bonding to the atom of interest (in this case N) is affected (e.g., if transport is carrier-mediated and if there is interaction between the N atom and the carrier). In general, isotopically sensitive steps are the catalytic ones, although, as previously mentioned, a prior equilibrium (as, e.g., $H^+ + NH_3 \leftrightharpoons NH_4^+$) can influence the isotopic composition of the product.

c. Degree of expression of intrinsic isotope effects In some reactions, more than one N-bearing substrate may enter the reaction, and, in others, the same substrate enters twice (as in denitrification, the process whose unique feature is the biological conversion of NO_2^- to N_2O). Individual steps within an overall reaction may or may not be reversible. The intrinsic

Table I
The Isotope Effect Associated with N_2-Fixation[a]

Host	Number of species	Mean observed isotope effect (β_{obs})
Azotobacter	4	1.0012
Glycine max		1.0015
Medicago sativa		0.9998
Trifolium	2	0.9995
Vicia faba		0.9998
Lupinus	2	1.0000
Phaseolus vulgaris		1.0015
Cyamopsis tetragonoloba		0.9992
Dalea	2	1.0017
Prosopis glandulosa		1.0015
Lotus pendunculatus		1.0001
Macroptillium atropurpureum		1.0034

[a] Summary of data published by Bergersen and Turner (1983), Delwiche and Steyn (1970), Domenach and Corman (1984), Hoering and Ford (1960), Kohl and Shearer (1980), Mariotti *et al.* (1980), Shearer *et al.* (1983), Shearer and Kohl (1986), Steele *et al.* (1983).

isotope effect associated with steps beyond the first irreversible step following the final entry of the N-bearing substrate cannot be expressed in β_{obs}, because all of the intermediate produced in that step is consumed in the forward direction. Therefore, all molecules, regardless of their isotopic composition, will be converted to product. Moreover, to the degree that the rate of reaction of any step in the reverse direction is slow, compared to the overall rate in the forward direction, the effect of isotopic discrimination associated with later steps will be moderated. For these reasons, β_{obs} associated with certain overall reactions is much smaller than might be expected. For example, one might expect a very large isotope effect for N_2 fixation because there is a very large change in bonding to N during reduction of dinitrogen to ammonia; however, this is not observed. Table I presents a summary of data collected from a number of laboratories on the magnitude of this isotope effect. In general, the isotope effect associated with this process is quite modest—usually less than about 1.002, or 2‰. Hoering and Ford (1960), who made the earliest measurements of this kind, suggested that the rate of N_2 fixation was limited by the rate of enzyme–substrate association; i.e., they proposed that the rate of enzyme–substrate dissociation is very slow compared to the overall rate, so that the intrinsic isotope effect associated with the reductive step is expressed only to a very moderate degree. Another possibility is that diffusion of N_2 to the enzyme is rate-limiting.

d. Variability of β_{obs} for a given reaction sequence The relative rates of forward and reverse steps in a reaction sequence are determined by the rate constants for the forward and reverse steps in question and the concentration of secondary substrates, the enzyme, or cofactors. To the degree that the concentration of molecules other than the nitrogenous substrate enter the reaction at different steps, the relative rates of forward and reverse steps in a reaction sequence may vary with environmental conditions that affect their concentrations. In this case, β_{obs} can be expected to vary. Such variation has been experimentally observed in the case of NO_3^- assimilation in pearl millet. Mariotti *et al.* (1982) reported a value of 1.027 for β_{obs} for NO_3^- assimilation in pearl millet seedlings. This isotope effect diminished with time during the growing season to a value approaching 1.000. The variability of this isotope effect was interpreted as follows. In the young seedlings, the concentration of nitrate reductase limits the overall rate of assimilation, so that not all of the NO_3^- entering the cell is reduced. That portion which is reduced is lighter than unreacted substrate, so that the intrinsic isotope effect associated with the reductive step is expressed. As the plants mature, nitrate reductase concentration increases to the point that all of the NO_3^- entering the cell is reduced, and the intrinsic isotope effect associated with reduction is no longer expressed. The lack of expression of the intrinsic isotope effect associated with NO_3^- reduction in more mature plants seems to be a general phenomenon. Mariotti *et al.* (1980) measured the ^{15}N abundance of 38 species of 2-mo-old hydroponically grown plants whose source of N was NO_3^- supplied in the medium. The $\delta^{15}N$ values of plant N were very close (on average, within 0.25‰) to that of the supplied NO_3^-; i.e, β_{obs} was approximately equal to 1.000.

The overall, observed isotope effect associated with dissimilatory NO_3^- and NO_2^- reduction has also been shown to vary with environmental conditions, namely, with the concentrations of the carbon source and NO_3^- or NO_2^- (Chien *et al.*, 1977; Mariotti *et al.*, 1981; Bryan *et al.*, 1983; Shearer and Kohl, 1988). Variation of β_{obs} for NO_2^- reduction, in combination with independent data on the relative rates of forward and reverse steps of the reaction, has been used to rule out the operation of one of two proposed mechanisms for dissimilatory nitrite reduction in *Pseudomonas stutzeri* (strain JM 300) under the conditions of the experiments (Shearer and Kohl, 1988).

e. Effect of the relative rates of reactions competing for the same substrate on the ^{15}N abundance of products Even if the actual value of β_{obs} is invariant with environmental conditions, the "apparent" value of β_{obs} can vary in complex systems in which more than one reaction competes for the same substrate, provided that the value of β_{obs} is different for the competing reactions. In soil systems, the ammonium ion occupies a central position in the N cycle as the substrate for nitrification, immobilization (the incorporation of inorganic soil N into biomass and thence into complex organic compounds of the soil), and volatilization. A model predicting the effect of varying the relative rates of nitrification and immobilization on the ^{15}N abundance of NO_3^-, NH_4^+, and available soil organic N has been put forward (Shearer *et al.,* 1974). According to this model, if nitrification is the only fate of NH_4^+, and if there is no isotopic fractionation associated with further reactions of NO_3^-, its ^{15}N abundance will be the same as N being mineralized from the available soil organic pool. As the rate of immobilization of NH_4^+ increases relative to the rate of nitrification, the ^{15}N abundance of NO_3^- will decrease (assuming that isotopic fractionation associated with nitrification is larger than that associated with immobilization, as all available data suggest is the case). If immobilization is the only fate of NH_4^+, the ^{15}N abundance of N being immobilized into the available soil organic N pool ^{15}N abundance will be the same as N being mineralized from the available soil organic pool and will increase as the relative rate of nitrification increases. This suggests another mechanism (in addition to NH_3 volatilization and denitrification) for increasing the ^{15}N abundance of soil organic N.

In the following sections, we discuss methods of measuring the natural abundance of ^{15}N and procedures for using variations in the natural abundance of ^{15}N in estimating the relative contribution of two sources to a common sink and in studying the mechanism of N transformations.

II. METHODS OF MEASURING THE NATURAL ABUNDANCE OF ^{15}N

Of the methods that have been used to measure N isotope composition, only mass spectrometry is sufficiently precise for measurements at the level of natural abundance. Chapter 2 of this volume covers the topic of mass spectrometry. Here we note only that an isotope-ratio-type mass spectrometer equipped with a dual collector (so that ions of masses 28 and 29 may be collected simultaneously) is required to minimize the effects of temporal instability. A dual-inlet system with changeover valve is also highly desirable, so that sample and reference gases can be measured alternately and repeatedly. Dual-collector, dual-inlet mass spectrometers suitable for measuring the natural abundance of ^{15}N have been commercially available for a number of years. The major improvement in the last few years is reduction of the amount of sample required, by trapping pure N_2 gas on molecular sieves or silica gel to reduce the volume of the gas and by the introduction of low-volume valving systems. Techniques for automation of the preparative stream, which have been successful for samples enriched in ^{15}N, are on the point of being improved sufficiently for use with samples at natural ^{15}N abundance. These sorts of improvements will increase the feasibility of large-scale studies in the future.

A. Sample Preparation

1. General Comments

The most important rules to follow in preparing samples for mass spectrometric analysis are the following. (i) Quantitative conversion is required at all steps of the procedure to avoid the effect of isotopic fractionation associated with incomplete conversion or with N loss. (ii) All steps of sample preparation should be done in areas and with equipment reserved for samples at natural ^{15}N abundance, because even the slightest contamination with ^{15}N-enriched material causes natural abundance data to be meaningless. Our practice is to borrow someone else's laboratory and glassware on the rare occasions we use ^{15}N-enriched material. (iii) It is necessary to obtain a representative subsample of the material to be analyzed. This requires that nonhomogeneous samples, such as soils, plant, or animal materials be finely pulverized. (iv) All preparations should be done in duplicate, at a minimum, and isotope analysis should be done on each duplicate, with replicate samples separately entering the preparative stream at the earliest practical time. This procedure allows for a statistical estimate of measurement error, *including* that introduced in sample preparation, and also informs the investigator if something has gone awry during sample preparation. Partial loss of sample N, which may have escaped the investigator's attention, becomes apparent when replicate determinations of the quantity of N or its ^{15}N abundance disagree. It is helpful to prepare and analyze replicate samples on different days to avoid systematic error. (v) Because high precision, as indicated by good analytical replicability, does not eliminate the possibility of systematic error, it is advisable to validate the methods being used by interlaboratory comparisons of the ^{15}N abundance of a few samples representative of the kind to be used in the study. Most laboratories engaged in measuring ^{15}N at the level of natural abundance are willing to participate in such interlaboratory comparisons.

It is theoretically possible to measure the isotopic composition of any nitrogenous gas such as N_2O; however, this would be needlessly complicated for most applications for two reasons. First, CO_2, which has the same mass as N_2O, and thus interferes with isotope analysis, cannot be separated cryogenically from N_2O but must be rigorously excluded by some other means. Second, the presence of three stable isotopes of oxygen (^{16}O, ^{17}O, and ^{18}O) greatly complicates data interpretation. (One application that might justify the complexity of measuring the isotopic composition of N_2O comes to mind. That application is estimating the relative contributions of anthropogenic vs. biogenic sources of N_2O to the atmosphere.) The gas most commonly introduced into the mass spectrometer for isotope analysis is N_2. It is therefore necessary to convert sample N to N_2 gas.

2. Total N

There are two methods for converting the total N of a sample to N_2. The Dumas method consists of combustion of the dehydrated sample in an evacuated, sealed quartz tube. If properly done, the product gases are N_2 and CO_2, which may be separated either cryogenically or some other way. The pure N_2 is then introduced into the mass spectrometer for isotope analysis. In the second method, Kjeldahl digestion, sample N is first converted to NH_4^+ by digestion in hot H_2SO_4 in the presence of a catalyst. The resulting NH_4^+, after being separated from the digest by steam distillation, is oxidized with hypobromite, under vacuum, to N_2, which is then entered into the mass spectrometer. The best evidence is that with proper care, either method is suitable for natural ^{15}N abundance determinations. Advantages and disadvantages of each method follow.

a. The Dumas method

i. Advantages This method is considerably less labor-intensive than the Kjeldahl method and more amenable to automation. Moreover, laboratories interested in measuring both ^{13}C abundance and ^{15}N abundance can use the same preparative setup for measurement of both isotopes and even the same combusted sample. Because of these compelling advantages, most laboratories setting up to measure the natural abundance of ^{15}N will choose this method.

ii. Disadvantages Incomplete combustion results in contaminating gases, including CO, which has the same mass as N_2 and cannot be cryogenically separated from N_2. Contamination with CO poses a particular problem for samples with a high C : N ratio. The smallest trace of CO will cause a significant artifact in the result of the isotope analysis. Because the natural abundance of ^{13}C is approximately threefold higher than that of ^{15}N, inclusion of even small amounts of CO in the sample will cause the ^{15}N abundance to appear larger than it actually is. Unfortunately, there is no way of knowing whether or not contaminating CO is present unless a gas chromatography apparatus is used in tandem with the mass spectrometer to detect any CO that may be present and separate it from N_2. Presently available commercial equipment that combines gas chromatography with isotope ratio mass spectrometry is not sufficiently sensitive to allow determination of variation in natural ^{15}N abundance. Until such time that gas chromatography–mass spectrometry instruments suitable for measuring natural ^{15}N abundance are available, it will be necessary to verify the Dumas combustion conditions to exclude, to the degree possible, contamination of N_2 with CO. This can be done in one of several ways: for example, (i) determine any apparent alteration in the ^{15}N abundance of standards of known $\delta^{15}N$ values after combusting them in the presence of N-free carbonaceous material, or (ii) compare $\delta^{15}N$ values of combusted samples with values determined on samples prepared by Kjeldahl digestion. Such a comparative study was done by Minagawa *et al.* (1984) on samples ranging in complexity from $(NH_4)_2SO_4$ to kerogen, with fairly good agreement. The mean difference was only 0.22‰. Only 1 of 11 samples showed a significant difference in $\delta^{15}N$ values ($p < 0.05$) and this difference was only 0.66‰. The more complex samples were slightly higher in both $\delta^{15}N$ and %N in samples prepared by Dumas combustion. The authors concluded that the Dumas method was superior because of the higher N yield. However, because N recovery in combusted samples was measured manometrically with cryogenically purified gases, it is also possible that the higher apparent N recoveries in combusted samples were the result of contaminating gases, namely, CO, which would also cause higher apparent $\delta^{15}N$ values. Although there were slight differences between the two methods in this study, the differences were so small as to be inconsequential. However, it cannot be assumed that different Dumas combustion routines will necessarily show equally good agreement, and it cannot be assumed that equal success will be achieved with samples of different character. For example, no soil samples were included in the study of Minagawa *et al.* (1984).

The procedure recommended is that of Minagawa *et al.* (1984). Details are found in Chapter 6.

b. Kjeldahl method

i. Advantages The major advantage of the Kjeldahl method is that all volatile contaminants, other than air introduced by leakage, can be removed cryogenically prior to introducing N_2 derived from the sample into the mass spectrometer. Contamination of the sample with air can be detected by routine mass spectrometric measurement of argon on each sample.

ii. Disadvantages The major disadvantage of this method, other than its labor-intensivity, is that it results in loss of NO_3^-. Although modifications are available in which NO_3^- is reduced, by thiosulfate or reduced iron, to NH_4^+ prior to digestion (Bremner, 1965), in our hands these modifications do not result in quantitative conversion of NO_3^- to NH_4^+, with the result that N derived from NO_3^- is isotopically altered. On the other hand, organic samples may have enough endogenous reductant to reduce NO_3^- partially, again resulting in isotopic alteration of the N derived from NO_3^-. For this reason, it is our practice to extract inorganic N from samples likely to contain significant amounts of NO_3^- (e.g., certain soil samples) and analyze this N separately (see Section II.A.3). For most plant samples, this step is unnecessary. If in doubt, we extract the plant tissue by grinding in a glass tissue grinder with 50 m*M* phosphate buffer (pH 7) and analyze the extract for NO_3^-.

iii. Extraction of inorganic N from soil samples The method for extraction of inorganic N is essentially that of Bremner and Keeney (1966). This method makes use of a strong salt that extracts exchangeable NH_4^+ as well as NO_3^-. Inorganic N is extracted from the soil sample by stirring or shaking one part by weight of soil with 10 volumes 2 *N* KCl for 1 hr. For the Kjeldahl procedure we use, 1 g soil is near the limit. If more soil is needed for isotope analysis, two or more 1-g samples are used and Kjeldahl digests pooled. The soil slurry is filtered through washed, ashless filter paper, and the material remaining on the filter paper is washed with additional 2 *N* KCl. This material is then quantitatively transferred to a Kjeldahl flask or tube.

iv. Digestion, distillation, and evaporation These procedures are described in Chapter 2, Section III.B. Some loss of NH_3 during distillation occurs unless the tip of the condenser is completely submerged under the surface of the collecting mixture. Because isotopic discrimination accompanies this loss, it is necessary to submerge the condenser tip for samples to be analyzed for N isotope abundance at the level of natural abundance. It is also necessary to avoid subjecting dry $(NH_4)SO_4$ samples to temperatures higher than 55°C during and after evaporation.

3. Inorganic N

If inorganic N is to be analyzed independently of organic N, three methods are available. Method 1 is based on that of Bremner and Keeney (1965). Method 2 is based on the method of Varner *et al.* (1953). In both methods, NH_4^+ is collected by distillation under moderately alkaline conditions. Then NO_3^- and NO_2^- are reduced to NH_4^+ which is collected by distillation. Both methods were designed for smaller samples than those required for N isotope analysis at the level of natural abundance and must be scaled up. We have had good results with both methods. Method 3 is applicable only to NH_4^+ and was designed for measurement of the natural abundance of ^{15}N in samples containing very low concentrations of NH_4^+ (Velinsky *et al.* 1989).

a. Method 1 The method of Bremner and Keeney (1965) has the advantage that the same distillation unit used for Kjeldahl digests can be used without modification, if the still has a detachable sample chamber. It has the disadvantage that N derived from NO_2^- cannot be collected independently from N derived from NO_3^-. However, NO_2^- can be destroyed with sulfamic acid (Bremner and Keeney, 1965) so that N derived from NO_3^- can be analyzed independently of that derived from NO_2^-. In method 1, MgO is used to drive the equilibrium between NH_4^+ and NH_3 toward NH_3, and the NH_3 is steam-distilled into boric acid containing methyl red and bromocresol green. Following removal of NH_4^+ in this way, Devarda's alloy is used to reduce NO_3^- (and NO_2^- if present) to NH_4^+, which is collected by steam distillation. Both MgO and Devarda's alloy must be pretreated prior to distillation. MgO is fired for 2 hr at 600–700°C and cooled in a desiccator. The Devarda's alloy must be ball-milled to pass a 300-mesh screen in order to increase the surface area. On some occasions (e.g., as in the case of soil extracts), the volume of sample required for the recovery of about 0.5 mg N for mass spectrometry will be large. In this case, the capacity of the sample flask for the distillation and the distillation time will need to be increased.

b. Method 2 The method of Varner *et al.* (1953) has the advantage that N derived from NH_4^+, amides, NO_3^-, and NO_2^- can be collected independently. Its chief disadvantage is that reducing sugars interfere, necessitating chromatographic removal of organics from the sample prior to distillation. We find this step to be unnecessary in the case of soil extracts. A second disadvantage is that a different kind of distillation unit than that used for Kjeldahl digests is required, because NH_4^+ is collected by vacuum distillation. A slight modification of the still, which requires no special equipment or glassblowing, will serve the purpose. A hole can be drilled in a stopper through which the tip of the condenser can be fitted. Tygon tubing can be connected to the condenser tip to lengthen it sufficiently to reach the bottom of a side arm flask that will be inserted onto the stopper. The side arm can be connected to an aspirator for evacuation of the system. This modified still can be used for the initial distillation of NH_4^+. After adding the sample to the sample chamber, and boric acid with mixed indicators to the side arm flask, saturated sodium tetraborate adjusted to pH 10 is added. The sample is heated to 54°C and NH_3 is trapped in the boric acid–mixed indicator solution by vacuum distillation. The force with which vacuum is pulled on the collecting flask must be low enough that NH_3 is effectively trapped by the boric acid. Quantitative NH_4^+ recovery can be checked by distilling a known quantity of a standard NH_4^+ solution. When this distillation is complete, NaOH is added, and NH_4^+ hydrolyzed from amides is collected by steam distillation. Following this step, NO_2^- (if present) is reduced to NH_3 with 20% $FeSO_4$ (w/v), and the NH_3 is collected by steam distillation. Finally, NO_3^- is reduced to NH_3 with saturated Ag_2SO_4, and the NH_3 is collected by steam distillation.

*c. **Method 3*** Method 3 (Velinsky *et al.*, 1989) was designed for measuring the natural abundance of ^{15}N of NH_4^+ in estuarine waters and is applicable to other kinds of environmental samples in which NH_4^+ concentrations are very low, such as rain water. While both methods 1 and 2 can be used for preparing samples with low concentrations of inorganic N, these methods require that the samples be concentrated. This presents a particular problem in the case of NH_4^+ because the samples must be acidified prior to evaporation to avoid volatile NH_3 loss. The method of Velinsky *et al.* (1989) has the advantage that the samples are distilled directly, in a high-volume steam still, with the NH_4^+ in the distillate being collected by ion exchange on zeolite (Union Carbide W-85 molecular sieve), which can then be collected by filtration. After drying, the NH_4^+ on the zeolite is then converted to N_2 by the Dumas method. A rather large error in the measured $\delta^{15}N$ value was observed for very small samples (Velinsky *et al.*, 1989). For example, an $(NH_4)_2SO_4$ standard with a $\delta^{15}N$ value of 20.73‰ yielded a $\delta^{15}N$ value 1.69‰ lighter when subjected to the procedure. However, when the sample size was increased to 200 μg N, the measured value was closer to the true value (within 0.7‰). The improvement with sample size indicated contamination with blank N of different isotope abundance. Samples of up to 500 ml are distilled from a 1.5-liter sample flask into a 2-liter receiving flask containing 20 ml 0.003 *N* HCl and 50–70 mg zeolite, which is preheated at 160°C for 1 hr. The contents of the receiving flask are stirred slowly with a stirring bar throughout the distillation. Immediately prior to distillation, the sample is adjusted to pH 9.5 with 6 *N* NaOH. MgO could be substituted for NaOH to avoid breakdown of labile organic matter during distillation (Bremner and Keeney, 1966). The distillation is carried out for 38 min at a rate of 13.9 ml/min. After the distillation is complete, the contents of the receiving flask are stirred for an additional hour, after which the zeolite is collected onto a Whatman GFF glass fiber filter that had been preheated to 420°C for 1 hr to remove contaminants. After drying under a stream of nitrogen at 50°C for 48 hr, the NH_4^+ is converted to N_2 by Dumas combustion.

4. N_2 Generation from $(NH_4)_2SO_4$

N_2 generation from NH_4^+ by oxidation with NaOBr or LiOBr under vacuum, as described in Chapter 2, Section III.C.

B. Isotope Analysis

The principles of isotope-ratio mass spectrometry are given in Chapter 2. For measurements at natural ^{15}N abundance, a dual collector mass spectrometer is required to measure $^{29}N_2$ and $^{28}N_2$ simultaneously. A number of corrections must be applied to determine the absolute ratio of $^{29}N_2$ to $^{28}N_2$ (e.g., a correction must be made for small differences in amplification of the currents from the two collectors). These correction factors cancel when the isotope ratios of two gases are being compared. For this reason a dual-inlet system is highly desirable so that sample and standard gases may be admitted alternately to the analyzer system of the mass spectrometer. The standard may be atmospheric N_2 purified according to methods described, for example, by Mariotti (1983) or a laboratory standard whose isotope abundance compared to atmospheric N_2 is known. Unpurified air is an inappropriate standard even if condensibles are removed with liquid N_2; its high O_2 concentration interferes with N isotope analysis and damages the filament. Provided that the two isotopes are randomly distributed, $\delta^{15}N$ values can be calculated from the measured values of $^{29}N/^{28}N$ of the sample and standard gas. The standard error of $\delta^{15}N$ calculated from six alternate measurements of sample and standard gases, with each measurement integrated over a period of 30 sec should be no greater than about 0.05‰. To determine the degree of photochemical breakdown of NaOBr, the O_2 peak is measured on the first sample of the day. Contamination of the sample with atmospheric N_2 is determined by measuring the argon peak on each sample.

III. APPLICATIONS

A. Source Identification

Differences in the natural abundance of ^{15}N have been used to estimate the relative contribution of two sources to a common sink—for example, sources of N to N_2-fixing symbiotic associations (e.g., Shearer and Kohl, 1986), sources of nitrate in surface water (Kohl *et al.*, 1971) and groundwater (e.g., Kreitler and Jones, 1975), sources of N in animal (e.g., Schoeninger and DeNiro, 1984) and human (e.g., Ambrose and DeNiro, 1986) diets. However, great care must be exercised in planning the sampling approach and interpreting the data. The primary problem is that isotopic discrimination associated with various N transformations can result in isotopic alteration of source N as it is transformed to sink.

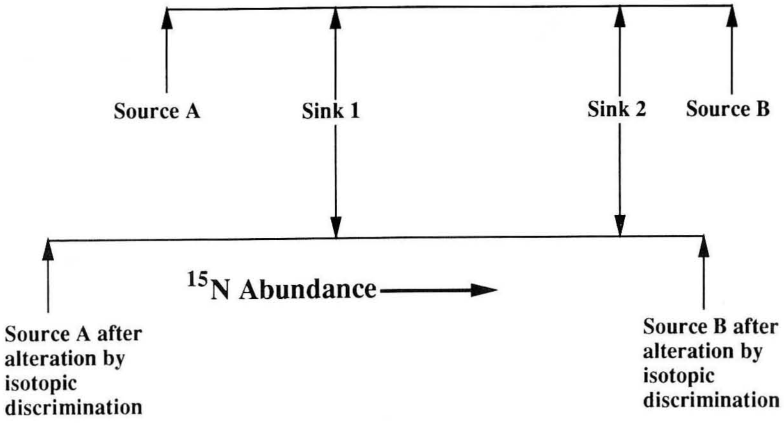
Source A
Sink 1
Sink 2
Source B
15N Abundance
Source A after alteration by isotopic discrimination
Source B after alteration by isotopic discrimination

Figure 1 Illustration of the principle of the natural ^{15}N abundance method for estimating the fractional contribution of one of two sources (Source A and Source B) to a common sink (Sink 1 or Sink 2). The ^{15}N abundance of both sources is lowered by isotope discrimination as they are transformed to the sink. The ^{15}N abundances of both sinks lie between those of the two sources, but the ^{15}N abundance of Sink 1 is closer to that of the isotopically altered Source A than to that of the isotopically altered Source B, indicating that Source A contributes more N to Sink 1 than does Source B. In contrast, Source B makes a larger contribution to Sink 2 than does Source A. A quantitative treatment is given in the text.

1. Principles

a. Estimating the relative contribution of two sources of N to a common sink The principle of estimating the relative contribution of two N sources to a common sink is similar to that of isotope dilution using ^{15}N-enriched materials and is illustrated in Fig. 1. The upper horizontal line of Fig. 1 shows what the situation would be in the absence of isotopic discrimination and the lower line takes into account the effect of discrimination that alters the ^{15}N abundance of the source as it is transformed to sink. In Fig. 1, it can be qualitatively concluded that, regardless of whether the upper or lower line is used, both sources contribute N to both sinks and that the contribution of Source A to Sink 1 is greater than it is to Sink 2. However, an estimation of the relative magnitude of these contributions of Source A to Sink 1, compared to that of Source B, depends heavily on whether or not isotopic discrimination is taken into account.

For example, suppose the following:

The $\delta^{15}N$ value of Source A ($\delta^{15}N_{\text{Source 1}}$) is 0‰.

The $\delta^{15}N$ value of Source B ($\delta^{15}N_{\text{Source 2}}$) is 10‰.

The $\delta^{15}N$ value of Sink 1 ($\delta^{15}N_y$) is 3‰.

The $\delta^{15}N$ value of Source A is lowered by 2‰ as a result of isotope discrimination; hence, the $\delta^{15}N$ value of N derived from Source A ($\delta^{15}N_c$) is −2‰.

The $\delta^{15}N$ value of Source B is lowered by 0.5‰ as a result of isotope discrimination; hence, the $\delta^{15}N$ value of N derived from Source B ($\delta^{15}N_x$) is 9.5‰.

The fractional contribution of Source A to Sink 1 can be calculated from a linear interpolation between the poles representing the two sources. Equation (A3) (derived in the Appendix and shown below) provides an expression for doing this. If one takes into account isotopic discrimination, the percent of N in Sink 1 derived from Source A is

$$\%\text{N derived from source A} = \frac{\delta^{15}N_x - \delta^{15}N_y}{\delta^{15}N_x - \delta^{15}N_c} \cdot 100 = 57\%.$$

Without taking into account isotopic discrimination, one would estimate that 70% of the N in the Sink 1 was derived from Source A and 30% from source B. The closer the ^{15}N abundance of the two sources, A and B, the more important it becomes to take isotopic discrimination into account. For example, had the $\delta^{15}N$ value of source A been 4.5‰, and the effect of isotopic discrimination to lower this value by 2‰, so that $\delta^{15}N_c = 2.5$‰, then the calculated value of %N dfa, taking into account isotope discrimination, would be 93% but the physically impossible value of 127% if isotopic discrimination were ignored.

Figure 1 highlights the following requirement for estimating the relative contribution of two N sources to a common sink: It is necessary that the ^{15}N abundance of the N of sources *after* they have undergone isotopic discrimination associated with its transformation to sink N be *measurably different*.

b. The variability of the estimate of the relative contribution of two sources of N to a common sink Not only is there measurement error associated with determination of N isotope abundance, there is also real variability in ^{15}N abundance of sources and sinks. This variability introduces uncertainty into estimates of the contribution of N from one of the sources to the sink. Therefore, reliable estimates cannot be made on the basis of one or a few measurements of the ^{15}N abundance of sink N and the N derived from two sources. A method of calculating the standard error of the estimate of the contribution of biologically fixed N to N_2-fixing plants, which includes both measurement error and real variability, was described by Shearer and Kohl (1986, eq. 5). Unfortunately that equation contains a mistake in sign. Equation (3) below is a corrected version of that equation. Equation (A3) (see Appendix) is of the form

$$F = 100 \cdot \frac{x - y}{x - c}$$

where F is the percentage of N in the sink of interest contributed by Source A, x is the δ^{15}N value of N derived from Source B, y is the δ^{15}N value of the sink, and c is the δ^{15}N value of N derived from source A.

$$\begin{aligned} \mathrm{SE}_F = 100 \cdot \Bigg[& \frac{(y - c)^2}{(x - c)^4}(\mathrm{SE}_x)^2 + \frac{1}{(x - c)^2}(\mathrm{SE}_y)^2 \\ & + \frac{(x - y)^2}{(x - c)^4}(\mathrm{SE}_c)^2 - \frac{2r_{y;c}(x - y)}{(x - c)^3}(\mathrm{SE}_y)(\mathrm{SE}_c) \\ & + \frac{2r_{x;c}(y - c)(x - y)}{(x - c)^4}(\mathrm{SE}_x)(\mathrm{SE}_c) \\ & - \frac{2r_{x;y}(y - c)}{(x - c)^3}(\mathrm{SE}_x)(\mathrm{SE}_y) \Bigg]^{1/2} \end{aligned} \qquad (3)$$

where r is the correlation coefficient for the indicated variables. The last three terms of the equation are covariance terms and may be neglected unless there is significant correlation between the indicated variables.

It should be noted that this error expression includes random error and real variation but does not include systematic error and error introduced through a badly designed sampling strategy. With these caveats in mind, Eq. (3) may be used to set limits on the estimates made from Eq. (A3). Equation (3) emphasizes another requirement for using natural ^{15}N abundance to estimate the relative contribution of N sources to a common sink, namely, the difference in ^{15}N abundance between N entering the sink from two sources *must be significant*.

c. Estimating the relative contribution of more than two sources of N to a common sink This is a much more complex problem than is the case when there are only two significant sources and will be treated here only very briefly. With only two isotopes, it is very difficult to distinguish among three sources of N, even if the differences in their ^{15}N abundances are large. If one or more of the sources is a point source, then information might be obtained by measuring the change in ^{15}N abundance of the sink as a function of distance from the source. Another possibility is to use two elements to distinguish sources. For example, in the case of determining sources of N_2O, variation in abundance of isotopes of both N and O are available. We do not know of any case in which multiple N-containing sources have been distinguished in this way, but examples exist for other isotopes. For example Craig and Craig (1972) were able to identify the probable origin of certain Greek marble archeological samples on the basis of ^{13}C and ^{18}O abundances.

2. Symbiotic N_2 Fixation: An Illustration of the Use of Natural ^{15}N Abundance for Source Identification

The growing literature on natural ^{15}N abundance to estimate the relative contribution of atmospheric N_2 to N_2-fixing organisms has been previously reviewed (Shearer and Kohl, 1986). This method has been shown to compare favorably to other more standard methods, such as the acetylene reduction assay (e.g., Amarger *et al.*, 1979), differences in N yield between N_2-fixing and nonfixing lines of soybeans (e.g., Kohl *et al.*, 1980), and isotope dilution with ^{15}N-enriched fertilizer (e.g., Ledgard *et al.*, 1985b) in numerous tests conducted under field and laboratory conditions. These tests have mostly been done with legumes in agricultural settings, although the natural ^{15}N method has also been applied to legumes (Shearer *et al.*, 1983), nonlegumes (Shearer and Kohl, 1986), and marine organisms (Wada and Hattori, 1976) in natural systems. This method is particularly useful in natural ecosystems because of the difficulty of applying other methods. Because the exact approach will depend on the site, the following procedures are illustrative only. This illustration is for a natural woodland ecosystem in which the ^{15}N abundance of N derived from plant-available soil N (the major source of N to nonfixing plants at this illustrative site) varies systematically across the site.

a. Evaluation of the suitability of the site Not all sites are suitable for application of the natural abundance method of estimating N_2 fixation; i.e., a given site may not meet the basic requirement that there must be a significant difference in ^{15}N abundance of N derived from atmospheric N_2 and N derived from other sources (predominantly soil N in terrestrial systems). Before investing a great deal of effort in the site, it is useful to determine if this requirement is met at the site of interest. Because the isotope effect associated with N_2 fixation usually alters the ^{15}N abundance of atmospheric N_2 by no more than 2‰ (see Table I), and most often less than this, it may be initially assumed that the site is suitable if the ^{15}N abundance of soil N that is available for uptake by plants is significantly different from that of atmospheric N_2. The site can thus be evaluated by surveying the ^{15}N abundance of the former (as described later) and determining its variability.

b. Determining the ^{15}N abundance of plant-available soil N The most appropriate method is to measure the ^{15}N abundance of non-N_2-fixing reference plants, rather than that of plant-available soil N itself, for several reasons. First, by measuring the ^{15}N abundance of N after it has been incorporated into the plant, any isotopic discrimination associated with N uptake and assimilation is taken into account. Second, the plant N is exactly the N taken up by the plant, while mineral N or N mineralized during incubation is only a proxy for the N actually taken up. Third, the ^{15}N abundance of plant-available N may vary with depth, so that to estimate the ^{15}N abundance of N taken up by the plant would require detailed knowledge of what fraction of the total plant N is taken from each depth as well as numerous measurements of the ^{15}N abundance of plant-available N as a function of depth. Few measurements have been made of the ^{15}N abundance of plant-available soil N as a function of depth, but considerable evidence indicates that the ^{15}N abundance of total soil N can vary with depth. Finally, some evidence indicates that the ^{15}N abundance of mineral N varies with time during the growing season (Turner *et al.*, 1987). The ^{15}N abundance of the plant's N is a depth- and time-integrated, weighted average of the ^{15}N abundance of N taken up by the plant, after it is altered by discrimination.

Time and depth dependence of plant-available soil N implies a difficulty with all ^{15}N methods of estimating N_2 fixation, including isotope dilution using ^{15}N-enriched materials. The basic assumption of all such methods is that the ^{15}N abundance of N of reference plants is identical to that of soil-derived N in the N_2-fixing plants. If reference plants and N_2-fixing plants take soil N from different depths and at different times, the soil-derived N in the two kinds of plants may have a different ^{15}N abundance. Selection of appropriate reference plants, with respect to depth and timing of uptake of soil-derived N, for the N_2-fixing plants of interest is of considerable importance. A very labor-intensive, although elegant, method for evaluating the suitability of reference plants has been devised by Ledgard *et al.* (1985a); however, this method is not applicable to our hypothesized natural ecosystem, because it involves a series of experimental plots to which ^{15}N-enriched fertilizer is added at different levels of enrichment. In natural systems, one must resort to qualitative criteria to judge the suitability of reference plants. The reference plants should have rooting patterns and growth forms similar to the N_2-fixing plants of interest, and the two kinds of plants should have similar phenology. As qualitative criteria are not altogether reliable, we have adopted the strategy of collecting tissues from as many different kinds of reference plants, which meet the qualitative criteria mentioned earlier, as possible. This allows assessment of the variability of our estimate of the ^{15}N abundance of soil-derived N, and this variability is included in the overall standard error of the estimate of the relative contribution of fixed N to the N_2-fixing plant.

Because our hypothetical site is a woodland, it is not possible, at least on a routine basis, to collect the entire plant. The most appropriate tissue to be collected will be discussed later (Section III.A.2.d).

c. Determining the ^{15}N abundance of biologically fixed N The δ^{15}N value of fixed N is usually very close to that of atmospheric N_2 (Section I.B.2). However, the difference, though small, is often significant and must be taken into account in arriving at estimates of the fractional contribution of atmospheric N_2 to N_2-fixing plants. The value is obtained by measuring the ^{15}N abundance of the N_2-fixing plant of interest grown under conditions in which it is forced to depend on atmospheric N_2 as its sole N source, other than seed N. These conditions are achieved by growing the plants hydroponically with N-free nutrient medium. Plants should be grown until the contribution of seed N is insignificant compared to the total N in the plant. Otherwise, the measured δ^{15}N value of the plant will have to be corrected for input of seed N to arrive at a value of δ^{15}N for fixed N. A sample calculation for taking into account seed N is given in the Appendix. Several pots should be grown and plants analyzed separately to obtain the standard error of the measurement. Our practice is to measure foliar tissue, stems, roots, and nodules separately and to calculate the ^{15}N abundance of the entire plant from the weighted average of these values (see Appendix).

d. Sampling strategy Once the initial survey of the site is complete, and the δ^{15}N value of fixed N for the N_2-fixing plant of interest has been determined, samples may be collected from the site for estimating the contribution of fixed N to the N_2-fixing plant. In the case of our hypothetical site, we will have determined that there is systematic variation in δ^{15}N values of soil-derived N across the site. We will consequently expect δ^{15}N values of reference and N_2-fixing plants to covary unless all of the N in the N_2-fixing plants is derived from atmospheric N_2. To measure the degree of covariance, we will need to collect N_2-fixing and reference plants in pairs, with the reference plant and N_2-fixing plant in each pair being in close proximity. If our survey has shown significant variation in δ^{15}N values among different kinds of reference plants, the collection should be made in groups to include a number of kinds of reference plants rather than in pairs.

There is significant, although small, variation in ^{15}N abundance from tissue to tissue. For example, stem tissue was lower in ^{15}N abundance than foliar tissue by about 1‰ in three N_2-fixing species of woody desert plants and eight associated species of reference plants collected from two sites (Shearer and Kohl, 1986). Ideally, a representative sample of the entire plant would be analyzed. However, in the case of our hypothetical woodland site, this would not be possible. Leaves or stems (branch wood) are the most feasible tissues to collect. While either leaves or stems can be used, mixtures of stems and leaves should be avoided, because the proportion of

Table II

^{15}N Abundance of Foliar Tissue of *Prosopis glandulosa* and Reference Plants at Seven Desert Sites

	^{15}N abundance–$\delta^{15}N^a$				Significance of difference between *P. glandulosa* and reference plants
	Reference plants		*P. glandulosa*		
Site	*n*	‰^{15}N	*n*	‰^{15}N	$(p)^b$
Harper's Well	12	5.1 (0.5)	40	1.2 (0.2)	<0.001
Transect near Harper's Well	15	3.0 (0.6)	9	1.1 (0.5)	<0.005
Nude Wash	19	5.7 (0.3)	1	1.2 —	—
Clark Dry Lake	4	13.0 (0.4)	3	4.7 (0.7)	<0.001
Borrego Sink	5	6.9 (0.7)	3	2.0 (0.2)	<0.001
Carrizo Badlands	4	6.6 (0.6)	2	2.2 (0.2)	<0.001
Baja California, Mexico, near Cataviña	13	9.3 (0.5)	5	8.9 (0.3)	NS

[a] Numbers in parentheses are standard errors.

[b] Significance of the difference was based on a two-tailed *t* test for ungrouped data, except for the transect near Harper's Well, in which samples were grouped.

each will vary and this will affect the measured value of $\delta^{15}N$. The same tissue should be collected from reference and N_2-fixing plants.

Very young plants and immature tissues should be avoided, because the isotope effect associated with NO_3^- assimilation is more likely to be expressed in such tissues, as discussed in Section I.B.2.c. Although application of the method does not require that this isotope effect not be expressed, it does require that the isotope effect for NO_3^- assimilation be the same in N_2-fixing and reference plants. Because this assumption cannot be tested in the field, the best course is to collect plants in which the expression of this isotope effect is minimal.

e. Calculations Table II shows mean $\delta^{15}N$ values for reference and N_2-fixing plants obtained in a study of N_2 fixation by the woody legume, *Prosopis glandulosa*, at seven desert sites (Shearer *et al.*, 1983). Table II provides a sense of the inter- and intrasite variability that is typically encountered. At one of the sites, only one specimen of *P. glandulosa* was available, which was sampled only once. Therefore, the significance of the difference between $\delta^{15}N$ values of *P. glandulosa* and reference plants cannot be evaluated at this site. At a second site, there was no significant difference between *P. glandulosa* and reference plants in ^{15}N abundance. At the other five sites, the difference between $\delta^{15}N$ values of *P. glandulosa* and reference plants was statistically significant ($p < 0.005$). Equation (A3) (from the Appendix), reproduced here, can be used to estimate the relative contribution of atmospheric N to N_2-fixing plants from such data:

$$\%\text{Ndfa} = \frac{\delta^{15}N_x - \delta^{15}N_y}{\delta^{15}N_x - \delta^{15}N_c} \cdot 100$$

where %Ndfa is the percentage of N in the N_2-fixing plant that is derived from atmospheric N_2;and $\delta^{15}N_x$, $\delta^{15}N_y$, and $\delta^{15}N_c$, are mean $\delta^{15}N$ values of leaves of reference plants, neighboring N_2-fixing plants, and the N_2-fixing plants grown hydroponically with N-free nutrient medium. The most appropriate tissue to be used for the $\delta^{15}N$ value of fixed N ($\delta^{15}N_c$) is the same kind of tissue collected in the field to obtain $\delta^{15}N_x$ and $\delta^{15}N_y$. The standard error of the estimate can be calculated from Eq. (3). If samples were collected in pairs or groups, as they were along the ~8-km transect near Harper's Well, the first three terms and the last of Eq. (3) will be needed, with the fourth and fifth terms dropping out because the $\delta^{15}N$ value of fixed N, a constant, is correlated with neither the $\delta^{15}N$ value of reference nor N_2-fixing plants. Samples were not collected in groups at the other sites. When samples are not collected in groups, only the first three terms of Eq. (3) can be used, the last three dropping out. A sample calculation is given in the Appendix for hypothetical ^{15}N data on foliar tissues of N_2-fixing and reference plants along a transect at our hypothetical site, where there is systematic variation in ^{15}N abundance across the site. The illustrative calculation makes clear the advantage of collecting samples in pairs or groups in such cases.

3. Caveats, Criticisms, Controversy

The publication of our first natural ^{15}N abundance source identification study on the source of NO_3^- pollution of the Sangamon River in central Illinois (Kohl *et al.*, 1971), in which we concluded that the source of about half of this NO_3^- was fertilizer, was met by a storm of criticism and controversy (e.g., Hauck *et al.*, 1972). Far less criticism has attended other source identification studies. Nevertheless considerable skepticism remains. Broadbent *et al.* (1980) reported a wide range of $\delta^{15}N$ values for total soil N at each of five California sites and concluded that

> It is clear that the spatial variability in distribution of $\delta^{15}N$ values is of the same order of magnitude as the difference between soil and atmospheric N. The data support the conclusion reached by others that it is not feasible to trace biological events in soils by means of natural abundance of ^{15}N (p. 527).

We certainly agree that if the variability reported in this article were general, it would not be possible to use variations in the natural abundance of ^{15}N to estimate N_2 fixation. However, as we have pointed out previously (Shearer and Kohl, 1986), the variability reported by Broadbent, *et al.* (1980) is quite atypical. Standard deviations calculated from their data for samples from five locations (the largest of which was 1.2 ha) were, on average, 3.5 times greater than 120 soil samples collected from across the United States, including Alaska (Shearer *et al.*, 1978); 2.4 times greater than 58 soil samples collected from a 74,000-km area in central Saskatchewan (Karamanos *et al.*, 1981); 2.4 times greater than 58 samples collected from 11 diverse sites in California (Virginia, 1980); 4.5 times greater than 60 samples collected across New Zealand (Steele and Wilson, 1981); and 6 times higher than at 18 sites in Australia (Ledgard *et al.*, 1984). The report by Broadbent *et al.* (1980) highlights the necessity of establishing the suitability of a site for natural ^{15}-N abundance use in source identification, as discussed in Section III.A.2.a.

Bremner and Hauck (1982) also were not optimistic:

> the many sources of error in sample collection and processing and in data interpretation militate against use of measuring variations in natural ^{15}N abundance as a means of obtaining reliable information about N cycle processes in complex biological systems (p. 486).

Knowles (1980) was similarly pessimistic about the value of the approach.

Critics and proponents of the natural ^{15}N abundance method of estimating N_2 fixation generally agree that the main problems that must be confronted are (i) the differences in isotopic composition being measured are extremely small; (ii) there is a degree of uncertainty involved in taking the effect of isotopic discrimination into account; and (iii) the ^{15}N abundance of soil N sources for the N_2-fixing and reference plants may not be identical. The adequacy of the strategies designed to respond to these problems has been tested in many greenhouse and field settings. Results from these tests have consistently provided estimates that vary in the expected direction in response to experimental manipulation and that compare favorably with other methods (for an extensive review of the evidence, see Shearer and Kohl, 1986). The results of these repeated tests lead us to consider estimates of N_2 fixation based on variations in the natural abundance of ^{15}N to be "semi-quantitative," a designation first proposed by Hauck (1973). The error of the natural ^{15}N abundance estimate is typically 5–10% (e.g., Shearer *et al.*, 1983). Because none of the more conventional methods can fairly be said to yield better precision (see, e.g., Shearer and Kohl, 1986), we consider the estimates of N_2 fixation based on natural ^{15}N abundance variations to be a useful approach. The method of choice will vary depending on the characteristics of the system being studied.

B. Turning a Sow's Ear into a Silk Purse: Isotopic Discrimination to Study Reaction Mechanism and Relative Rates of Steps within Reactions

The differences in ^{15}N abundance between N sources that make possible the source identification studies discussed earlier (Section III.A) are a result of the cumulative effects of isotopic discrimination over biological time. But isotope discrimination in N transformations are clearly an impediment to making precise estimates of the fractional contribution of two sources to a common sink. However, this same isotope discrimination that presents a problem for source identification can potentially reveal other important biological information. For example, dietary N becomes enriched in ^{15}N as it is assimilated into animal tissues (Miyake and Wada, 1967). This results in an increase in ^{15}N abundance with increasing trophic level (Schoeninger and DeNiro, 1984). The increase in ^{15}N from one trophic level to the next is "large enough to serve as an indicator of trophic level" (Ambrose and DeNiro, 1986). Variation in the magnitude of isotopic fractionation associated with a particular N transformation can also be used to study that transformation. The following discussion focuses on exploitation of variation in the expression of isotope discrimination to study N transformations.

1. Principles

The principle of the use of isotope effects for studying N transformations was alluded to in Sections I.A.2.b and I.A.2.c. Expression of intrinsic isotope effects associated with individual steps of a reaction sequence depend on the mechanism of the reaction and the relative rates of forward and reverse steps. Because the rate of any individual step depends on the concentration of all reactants, including secondary substrates and cofactors, the relative rates of forward and reverse steps may vary with environmental conditions, with a consequent variation in the overall, observed isotope effect, β_{obs}. This variation can provide information on the relative rates of forward and reverse steps, if the mechanism is known. If the mechanism is not known, but the relative rates of forward and reverse steps are, information about the mechanism of the reaction can be extracted from variation in β_{obs}.

This approach has been used to account for variation of ^{13}C abundance in plants with humidity and salinity (Farquhar *et al.*, 1982b; Winter *et al.*, 1982), based on a theoretical model of the relationship between ^{13}C abundance of leaves and internal CO_2 concentration (Farquhar *et al.*, 1982a), which depends on the ratio of efflux of CO_2 to its influx. On the basis of such a model and natural ^{13}C measurements, O'Leary and Osmund (1980) were able to conclude that carbon fixation by two species of CAM plants was limited principally by diffusion rather than by carboxylation.

We have used this approach in the study of denitrification of NO_2^- to N_2O by *Pseudomonas stutzeri* (Bryan *et al.*, 1983; Shearer and Kohl, 1988). Two types of mechanisms had been proposed for this reaction. In one type, two nitrite ions are reduced in parallel, with the N═N bond of N_2O being formed from reduced species (Payne, 1981; Garber and Hollocher, 1982). In the other type, the nitrite ions are reduced in a linear path, with the N═N bond being formed prior to reduction (Averill and Tiedje, 1982). We observed that β_{obs} for the reaction increased with NO_2^- concentration and decreased with the concentration of the stoichiometric reductant (Bryan *et al.*, 1983). This variation in β_{obs} did not, by itself, allow us to distinguish between these mechanisms, because we had no information about the relative rates of forward and reverse steps. However, good evidence indicated the existence of an early protonation–dehydration step (Garber and Hollocher, 1982), and this step was common to both mechanisms. This allowed us to investigate the relative rates of forward and reverse steps by determining the rate of incorporation of ^{18}O from water into NO_2^-. This rate was extremely slow compared to the forward rate of NO_2^- reduction. This information, in combination with the observed variation in β_{obs}, allowed us to rule out the operation of the first type of mechanism in this organism and under the conditions of our experiments (Shearer and Kohl, 1988).

The proposed mechanisms of denitrification of NO_2^- are rather complex, partly because of the entry of two nitrite ions in the reaction leading to the product, N_2O. We refer the interested reader to the original papers (Bryan *et al.*, 1983; Shearer and Kohl, 1988); in this chapter, we prefer to illustrate the methods used with a simpler, three-step model in which substrate in the medium, S_o, is transported into the cell in step 1. Inside the cell, the substrate, now designated S_i may be metabolized to product P (step 3), or it may be excreted from the cell (step 2) (see Fig. 2). An enzyme, E, is required for conversion of S_i to P. The reaction is in steady state; i.e., the rate of entry of S_o into the cell is equal to the sum of rates of disappearance of S_i—efflux from the cell plus metabolic conversion to P. No isotopic discrimination is associated with transport, unless the N atom of S_o or S_i forms a covalent bond with a carrier as it crosses the cell membrane. In the model of Fig. 2, the intrinsic isotope effects for steps 1 and 2 (β_1 and β_2) are equal to 1.000. However, the metabolic step ($S_i \rightarrow P$) is isotopically sensitive because the N atom is involved in the reaction; i.e., the intrinsic isotope effect for step 3 (β_3) is >1.000. The expectation is that β_3 will be expressed to the degree that step 1 is reversible (i.e., the rate of step 2 is >0). If step 1 is irreversible, β_3 cannot be expressed, because all of S_o entering the cell, regardless of whether S_o contains ^{15}N or ^{14}N, reacts in the forward direction. If the magnitude of β_3 is known, it is possible to determine the ratio of the overall rate of reaction in the forward direction to the rate in the reverse direction. Because the system is in steady state, the rate of step 1 is equal to the rate of step 2 plus the rate of step 3. Thus, the ratio of the rate of step 1 to the rate of step 2 (i.e., the ratio of influx of S_o into the cell to efflux of S_i out of the cell) can also be determined. Two procedures are needed. First it is necessary to measure β_{obs}. Then it is necessary to derive an expression for the relationship between the overall, observed isotope effect, β_{obs}, and the relevant relative rates.

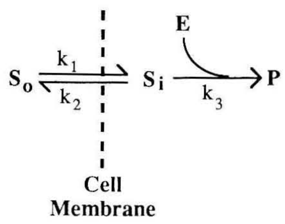

Figure 2 A simple three-step model for an enzymatically catalyzed reaction. The nitrogen-containing substrate in the medium outside the cell (S_o) enters the cell in step 1. Inside the cell, the substrate (S_i) is metabolized to product (P) by a reaction catalyzed by an enzyme (E) in step 3. Unreacted S_i exits from the cell in step 2. The rate constants (k_i) for each step are indicated by subscript. The ^{15}N abundance of S_i is increased by step 3, which discriminates against ^{15}N-bearing molecules. As S_i exits from the cell, it enriches the external substrate pool in ^{15}N. The degree of expression of the isotope effect associated with step 3 is determined by the rate of step 2 relative to the rate of step 3, as discussed in the text.

2. Experimental Determination of the Overall Isotopic Fractionation Factor, β_{obs}

a. Open systems In open systems, the substrate, of constant ^{15}N abundance, is either supplied at the same rate as it is consumed, as in a chemostat, or else it is present in amounts that are essentially infinite compared to the amount consumed, as in N_2 fixation. Under these conditions,

$$\beta_{obs} = \frac{(^{15}N/^{14}N)_{substrate}}{(^{15}N/^{14}N)_{product}}$$

This is essentially equal to

$$\beta_{obs} = \frac{(0.001\delta^{15}N + 1)_{substrate}}{(0.001\delta^{15}N + 1)_{product}} \qquad (4)$$

b. Closed systems In closed systems, substrate concentration declines during the reaction. The first increment of product made will be depleted in ^{15}N as a result of isotope discrimination, raising the ^{15}N abundance of the substrate. The next increment of product made will therefore have a somewhat higher ^{15}N abundance than the first increment because it is being made from a substrate with higher ^{15}N abundance. The result is that the ^{15}N abundance of both substrate and product will increase with extent of reaction, until the reaction is complete, at which time the product (or the weighted average of products, if there is more than one) will have the same ^{15}N abundance as the initial substrate. Therefore, the extent of reaction must be taken into account when determining β_{obs} from experimental data. Provided that β_{obs} is constant with time during the reaction, it may be calculated from the ^{15}N abundance of substrate at two time points, usually $t = t_0$ and $t = t_1$, where t_0 and t_1 represent time 0 and an intermediate time during the course of the reaction, as described by Mariotti *et al.* (1981). The ^{15}N abundance of product, rather than substrate, may be measured at t_1 because of the necessary relationship between ^{15}N abundance of substrate and product imposed by conservation of mass.

$$(\delta^{15}\text{N}_{p,t_1})(x) + (\delta^{15}\text{N}_{s,t_1})(y) = (\delta^{15}\text{N}_{s,t_0})(x + y)$$

where p,t_1, s,t_1, and s,t_0 are product at time 1, substrate at time 1, and substrate at time 0, respectively; and x and y are the amount of product at time 1 and the amount of substrate at time 1, respectively. An algebraically rearranged version of an exact expression of Mariotti *et al.* (1981) for the experimentally determined value of β_{obs} is

$$\beta_{\text{obs}} = \frac{\ln f}{\ln\left[\dfrac{0.001\delta^{15}\text{N}_{s,t_1} + 1}{0.001\delta^{15}\text{N}_{s,t_0} + 1}\right] + \ln f}$$

An approximate version of this equation, which gives a value of β_{obs} that agrees with the preceding one to well within experimental error was derived by Mariotti *et al.* (1981). A rearranged version of this approximation is

$$\beta_{\text{obs}} = 1 + \frac{\delta^{15}\text{N}_{s,t_0} - \delta^{15}\text{N}_{s,t_1}}{1000 \ln f} \tag{5}$$

3. Derivation of an Expression for the Relationship between the Overall, Observed Isotope Effect, $\boldsymbol{\beta_{\text{obs}}}$, and Relative Rates of Forward and Reverse Steps of a Reaction Sequence

The derivation begins by writing rate equations for ^{14}N bearing constituents of the reaction. The overall rate in the forward direction (v) is given by

$$v = \frac{d[P]}{dt} = k_3[S_i][E] \tag{6}$$

where k_3 is the ^{14}N rate constant for step 3.

The steady-state concentration of $[S_i]$ is derived as follows:

$$\frac{d[S_i]}{dt} = 0 = k_1[S_o] - [S_i](k_2 + k_3[E])$$

where k_1 and k_2 are the ^{14}N rate constants for steps 1 and 2. Therefore,

$$[S_i] = \frac{k_1[S_o]}{k_2 + k_3[E]}$$

Substituting this into Eq. (6) gives

$$v = \frac{k_1 k_3[S_o][E]}{k_2 + k_3[E]} \tag{7}$$

A similar set of equations can be written for ^{15}N bearing S_o, with the following result:

$$^*v = \frac{^*k_1 {}^*k_3[^*S_o][E]}{^*k_2 + {}^*k_3[E]} \tag{8}$$

where the asterisk refers to ^{15}N.

From Eq. (2), it can be seen that an expression for β_{obs} can be obtained by dividing Eq. (7) by Eq. (8). Note that in competitive experiments (those in which both isotopes are present in the same vessel) $[E]$ will be the same in Eqs. (7) and (8) and will cancel from the numerators of the two equations when one is divided by the other.

$$\beta_{\text{obs}} = \frac{v/[S_o]}{^*v/[^*S_o]} = \frac{k_1 k_3(^*k_2 + {}^*k_3[E])}{^*k_1 {}^*k_3(k_2 + k_3[E])}$$

The intrinsic isotope effect for step (i) is equal to $k_{(i)}/{}^*k_{(i)} = \beta_i$. Therefore this equation may be rewritten as

$$\beta_{\text{obs}} = \beta_1\beta_3\left[\frac{k_2/\beta_2}{k_2 + k_3[E]} + \frac{k_3[E]/\beta_3}{k_2 + k_3[E]}\right]$$

Because

$$\frac{k_2}{k_2 + k_3[E]} = 1 - \frac{k_3[E]}{k_2 + k_3[E]}$$

$$\beta_{\text{obs}} = \beta_1\beta_3\left[\frac{1}{\beta_2} - \frac{k_3[E]/\beta_2}{k_2 + k_3[E]} + \frac{k_3[E]/\beta_3}{k_2 + k_3[E]}\right], \text{ and}$$

$$\beta_{\text{obs}} = \frac{\beta_1\beta_3}{\beta_2} - \left[\frac{k_3[E]}{k_2 + k_3[E]}\right]\left[\frac{\beta_1\beta_3}{\beta_2} - \beta_1\right] \tag{9}$$

To obtain β_{obs} in terms of the ratio of the forward rate, v, to the sum of efflux (v_e) plus v, recall that $v = k_3[E][S_i]$, and $v_e = k_2[S_i]$. Therefore,

$$\frac{v}{v_e + v} = \frac{k_3[E][S_i]}{k_2[S_i] + k_3[E][S_i]} = \frac{k_3[E]}{k_2 + k_3[E]}$$

The ratio $v/(v_e + v)$ may be substituted into Eq. (9) to give

$$\beta_{\text{obs}} = \frac{\beta_1\beta_3}{\beta_2} - \left[\frac{v}{v_e + v}\right]\left[\frac{\beta_1\beta_3}{\beta_2} - \beta_1\right] \tag{10}$$

Because under steady-state conditions, $v = v_i - v_e$, where v_i represents influx,

$$\beta_{\text{obs}} = \frac{\beta_1\beta_3}{\beta_2} - \left[\frac{v_i - v_e}{v_e + v_i - v_e}\right]\left[\frac{\beta_1\beta_3}{\beta_2} - \beta_1\right]$$

$$= \frac{\beta_1\beta_3}{\beta_2} - \left[\frac{v_i - v_e}{v_i}\right]\left[\frac{\beta_1\beta_3}{\beta_2} - \beta_1\right] \tag{11}$$

$$\therefore \beta_{\text{obs}} = \beta_1 + \frac{v_e}{v_i}\left[\frac{\beta_1\beta_3}{\beta_2} - \beta_1\right]$$

Because β_1 and β_2 are equal to 1.000, if we know the magnitude of β_3, we can determine the ratio of efflux of substrate from the cell to influx of substrate into the cell. Then if the overall reaction rate is measured, we can determine the absolute values for influx and efflux. Even if the value of β_3 is unknown, if the measured value of β_{obs} varies with environmental conditions, the effect of the environmental change on the ratio of efflux to influx of substrate can be determined. In the case of the simple illustrative, three-step model used here (Fig. 2), variation in β_{obs} might occur if the free enzyme concentration varied. In other more realistic models, variation in the concentration of other reactants (e.g., the immediate reductant in the case of denitrification) might cause variation in β_{obs}. The study of Mariotti *et al.* (1982) showed that β_{obs} associated with NO_3^- assimilation by pearl millet declined as the plant matured. It can be concluded that the rate of NO_3^- efflux relative to influx decreased with plant growth. That is, as the plant matures, a greater fraction of the NO_3^- entering the cell is incorporated into plant protein. We have used this approach to study variation in the ratio of efflux to influx of NO_3^- in *Synechococcus* (Shearer *et al.*, 1991).

APPENDIX

Sample Calculation: δ^{15}N

Equation (1) of the main text (Section 1.A) defines δ^{15}N as

$$\delta^{15}\text{N} = \frac{R_{(\text{spl})} - R_{(\text{std})}}{R_{(\text{std})}} \cdot 1000‰$$

where R is customarily defined in one of two ways:

$$R = \frac{^{15}\text{N}}{^{15}\text{N} + {}^{14}\text{N}} \quad \text{or} \quad R = \frac{^{15}\text{N}}{^{14}\text{N}}$$

The difference in δ^{15}N values calculated from these two definitions of R is insignificant at natural abundance, as shown later, but becomes more important as δ^{15}N values increase. Suppose that a sample has a ratio of $^{15}\text{N}/(^{15}\text{N} + {}^{14}\text{N}) = 0.003736$, and the standard, atmospheric N_2, has a ratio of $^{15}\text{N}/(^{15}\text{N} + {}^{14}\text{N}) = 0.003663$. Substituting these values into the first definition of R, and the result into Eq. (1) gives a δ^{15}N value of 19.9‰, a rather large natural ^{15}N abundance. These ratios of $^{15}\text{N}/(^{15}\text{N} + {}^{14}\text{N})$ correspond to $^{15}\text{N}/^{14}\text{N}$ ratios of 0.003750 and 0.003676 for sample and standard, respectively. Substituting these latter values into Eq. (1) gives a δ^{15}N value of 20.0‰. The difference is well within experimental error. We conclude that these two definitions of R are essentially equivalent at the level of natural abundance.

Derivation of Isotope Dilution Equation in Terms of δ^{15}N

The N of a sink (y) is derived from two N sources, c and x. The δ^{15}N values of these sources after isotopic alteration due to discrimination as the sources are transformed are designated $\delta^{15}\text{N}_c$ and $\delta^{15}\text{N}_x$. The ratio of $^{15}\text{N}/(^{15}\text{N} + {}^{14}\text{N})$ of the sink (R_y) is equal to

$$R_y = \frac{^{15}\text{N}_c + {}^{15}\text{N}_x}{^{14}\text{N}_c + {}^{15}\text{N}_c + {}^{14}\text{N}_x + {}^{15}\text{N}_x} \tag{A1}$$

Equation (A1) can be rewritten as

$$R_y = \frac{^{15}\text{N}_c/c}{(x + c)/c} + \frac{^{15}\text{N}_x/x}{(x + c)/x} = \left[\frac{c}{x + c}\right]\left[\frac{^{15}\text{N}_c}{c}\right] + \left[\frac{x}{x + c}\right]\left[\frac{^{15}\text{N}_x}{x}\right]$$

where x is the amount of N in the sink derived from Source 2 and is equal to $^{15}\text{N}_x + {}^{14}\text{N}_x$, and c is the amount of N in the sink derived from Source

1 and is equal to $^{15}N_c + 14N_c$. Therefore, $^{15}N_x/x$ can be written as R_x and $^{15}N_c/c$ can be written as R_c. The preceding equation may be rewritten as

$$R_y = \left[\frac{c}{x + c}\right][R_c] + \left[\frac{x}{x + c}\right][R_x]$$

but $x/(x + c) = 1 - c/(x + c)$. Therefore,

$$R_y = \left[\frac{c}{x + c}\right][R_c] + R_x - \left[\frac{c}{x + c}\right][R_x]$$

$$\text{or}\quad R_y = \left[\frac{c}{x + c}\right][R_c - R_x] + R_x$$

From this equation, the fractional contribution of Source 1 $[c/(x + c)]$ can be written

$$\frac{c}{x + c} = \frac{R_x - R_y}{R_x - R} \tag{A2}$$

From Eq. (1) of the main text,

$$R = (0.001\delta^{15}\mathrm{N} + 1)\cdot R_{(\mathrm{std})}$$

Substituting this into Eq. (A2) gives

$$\frac{c}{x + c} = \frac{(0.001\delta^{15}\mathrm{N}_x + 1)\cdot R_{(\mathrm{std})} - (0.001\delta^{15}\mathrm{N}_y + 1)\cdot R_{(\mathrm{std})}}{(0.001\delta^{15}\mathrm{N}_x + 1)\cdot R_{(\mathrm{std})} - (0.001\delta^{15}\mathrm{N}_c + 1)\cdot R_{(\mathrm{std})}}$$

This equation simplifies to

$$\frac{c}{x + c} = \frac{\delta^{15}\mathrm{N}_x - \delta^{15}\mathrm{N}_y}{\delta^{15}\mathrm{N}_x - \delta^{15}\mathrm{N}_c}$$

The %N derived from Source 1 ($\%\mathrm{Ndf}_{\mathrm{source}_1}$) is obtained by multiplying the right side of this equation by 100:

$$\%\mathrm{Ndf}_{\mathrm{source}_1} = \frac{\delta^{15}\mathrm{N}_x - \delta d^{15}\mathrm{N}_y}{\delta^{15}\mathrm{N}_x - \delta^{15}\mathrm{N}_c}\cdot 100 \tag{A3}$$

Taking into Account the Contribution of Seed N

If plants are small, or seeds large, a significant fraction of the N of a plant may be derived from the seed. Suppose that the total N in a plant is 120 ± 8 mg (SE) and that there is an average of 20 ± 2 mg (SE) N in the seed. Suppose that the ^{15}N abundance of the total plant N is +3.1 ± 0.2‰ ^{15}N, and that of the seed 6.3 ± 0.4‰ ^{15}N. Assuming that all of the seed N has entered the plant, the $\delta^{15}N$ value of N in the plant that was derived from any source other than seed can be calculated from the mass balance equation

$$\delta^{15}\mathrm{N}_p\cdot x_p = \delta^{15}\mathrm{N}_s\cdot x_s + \delta^{15}\mathrm{N}_q\cdot x_q$$

where x_p is the quantity of N in the whole plant, x_s is the quantity of N derived from the seed, and x_q is the quantity of N derived from other sources.

$$\delta^{15}\mathrm{N}_q = \frac{\delta^{15}\mathrm{N}_p\cdot x_p - \delta^{15}\mathrm{N}_x\cdot x_s}{x_q} = \frac{\delta^{15}\mathrm{N}_p\cdot x_p - \delta^{15}\mathrm{N}_s\cdot x_s}{x_p - x_s}$$

In our example,

$$\delta^{15}\mathrm{N}_q = \frac{[3.1 \times 120] - [6.3 \times 20]}{120 - 20} = \frac{372 - 126}{100} = 2.46‰ \tag{A4}$$

The standard error of $\delta^{15}N_q$ (SE_q) is given by

$$\mathrm{SE}_q = \left[\frac{b^2}{(b - d)^2}(\mathrm{SE}_a)^2 + \frac{d^2(c - a)^2}{(b - d)^4}(\mathrm{SE}_b)^2 + \frac{d^2}{(b - d)^2}(\mathrm{SE}_c)^2 + \frac{b^2(a - c)^2}{(b - d)^4}(\mathrm{SE}_d)^2\right]^{\frac{1}{2}} \tag{A5}$$

where a is $\delta^{15}N_p = 3.1 \pm 0.2‰$, b is $x_p = 120 \pm 8$ mg, c is $\delta^{15}N_s = 6.3 \pm 0.4‰$, and $d = x_s = 20 \pm 0.2$ mg.

$$\mathrm{SE}_q = \left[\frac{[120]^2}{[120 - 20]^2}[0.2]^2 + \frac{[20]^2[6.3 - 3.1]^2}{[120 - 20]^4}[8.0]^2 + \frac{[20]^2}{[120 - 20]^2}[0.4]^2 + \frac{[120]^2[3.1 - 6.3]^2}{[120 - 20]^4}[2.0]^2\right]^{1/2}$$

$$= 0.25$$

Calculation of δ^{15}N Value of Entire Plant from δ^{15}N Values of Plant Parts

This calculation is similar to the one given earlier (Appendix, Section III) except that here the data can be grouped. That is, the $\delta^{15}N$ value of each entire plant may be calculated from its individual parts, and the standard error of the mean $\delta^{15}N$ value determined directly from values calculated for replicate whole plants. Suppose the plant is divided into the following parts: leaves (L), stems (S), reproductive tissues (F), roots (R), and nodules (N). The $\delta^{15}N$ value of the entire plant (E) is given by

$$\delta^{15}N_E = \frac{\delta^{15}N_L \cdot x_L + \delta^{15}N_S \cdot x_S + \delta^{15}N_F \cdot x_F + \delta^{15}N_R \cdot x_R + \delta^{15}N_N \cdot x_N}{x_L + x_S + x_F + x_R + x_N} \tag{A6}$$

where x is the quantity of N in the indicated tissue. For example, suppose the quantity of N in leaves, stems, reproductive tissues, roots, and nodules is 45, 25, 10, 30, and 10 mg, respectively, and $\delta^{15}N$ values of these tissues are +0.4, −0.6, −0.3, +0.2, and 8.0‰, respectively.

$$\delta^{15}N_E = \frac{[45 \times 0.4] - [25 \times 0.6] - [10 \times 0.3] + [30 \times 0.2] + [10 \times 8.0]}{45 + 25 + 10 + 30 + 10}$$

$$= 0.7‰.$$

Suppose that similar calculations for the next 4 replicate plants yielded values of $\delta^{15}N_E$ of 0.5, −0.5, −0.6, and 0.1‰, respectively. The mean value of $\delta^{15}N_E$ would be 0.04 ± 0.26%, where the error term is the standard error.

Sample Calculation of the Percentage of N in N_2-Fixing Plants Derived from Fixed N (%NDFA)

Table III gives hypothetical $\delta^{15}N$ data for foliar samples from N_2-fixing and reference plants along a transect running across our hypothetical site (see Section III.A.2 of the main text). At this hypothetical site, there is systematic variation in the ^{15}N abundance of soil-derived N across the site, with concomitant variation in $\delta^{15}N$ values of reference and N_2-fixing plants. This variation is so large that, on the basis of an unpaired t test, there is no significant difference in ^{15}N abundance between of the two kinds of plants. The mean value of reference plants is 5.35 ± 1.03‰ (SE), whereas the mean value for N_2-fixing plants is 4.01 ± 0.83‰ (SE). However, on the basis of a paired t test, the difference of 1.34‰ is significant at the 99.9% confidence level. Using Eq. (A3), the percentage of the N_2-fixing plant's N derived from atmospheric N_2 (%Ndfa) can be calculated from the mean $\delta^{15}N$ values of leaves from the reference and N_2-fixing plants from the hypothetical site and the $\delta_{15}N$ value of fixed N in foliar tissue of the same N_2-fixing plant grown in the greenhouse with N-free nutrient medium. The latter value is −1.1 ± 0.3‰. This value corresponds to the $\delta^{15}N$ value of Source 1; i.e., $\delta^{15}N = -1.1‰$. The mean value for reference plants corresponds to the $\delta^{15}N$ value of Source 2; i.e., $\delta 15N_x = 5.35‰$, whereas the mean value for N_2-fixing plants corresponds to the $\delta^{15}N$ value of the sink; i.e., $\delta^{15}N_y = 4.01‰$.

Table III
^{15}N Abundance of Foliar Tissue of N_2-Fixing and Reference Plants—Illustrative Data for Foliar Samples Collected along a Transect at a Hypothetical Site.

Sampling point	^{15}N abundance–$\delta^{15}N$ (‰15N)	
	Reference plants	N_2-fixing plants
1	−1.1	−1.0
2	1.5	1.1
3	4.3	2.6
4	3.9	3.1
5	5.3	3.6
6	7.4	5.8
7	6.8	5.9
8	7.9	5.3
9	8.3	7.2
10	9.2	6.5
Mean	5.35	4.01
SE	1.03	0.83

$$\%\text{Ndfa} = \frac{\delta^{15}N_x - \delta^{15}N_y}{\delta^{15}N_x - \delta^{15}N_c} \cdot 100 + \cdot \frac{5.35 - 4.01}{5.35 + 1.1} \cdot 100 = 20.8\%$$

In calculating the standard error of the estimate, the fourth and fifth terms of Eq. (3) of the main text drop out because they are covariance terms for $\delta^{15}N_x$ versus $\delta^{15}N_c$ and $\delta^{15}N_y$ versus $\delta^{15}N_c$, and there is no correlation between these variables. However, the correlation between $\delta^{15}N_x$ and $\delta^{15}N_y$ is quite strong, with a correlation coefficient (r) of 0.978. The relevant terms for calculating the standard error of %Ndfa are the first, second, third, and sixth terms of Eq. (3) (in the main text).

$$\text{SE}_{\%\text{Ndfa}} = 100 \cdot \left[\frac{(y - c)^2}{(x - c)^4} (\text{SE}_x)^2 + \frac{1}{(x - c)^2} (\text{SE}_y)^2 + \frac{(x - y)^2}{(x - c)^4} (\text{SE}_c)^2 - \frac{2 r_{x,y} (y - c)}{(x - c)^3} (\text{SE}_x)(\text{SE}_y) \right]^{1/2}$$

$$= 100\cdot\left[\frac{(5.11)^2}{(6.45)^4}(1.03)^2 + \frac{1}{(6.45)^2}(0.83)^2 + \frac{(1.34)^2}{(6.45)^4}(0.30)^2 \right.$$

$$\left. - \frac{[2 \times 0.978](5.11)}{(6.45)^3}(1.03)(0.83)\right]^{1/2}$$

$$= 2.9\%$$

The result is that the percent contribution of atmospheric N_2 to the N of the N_2-fixing plants is 20.7 ± 2.9%. Had the last term been neglected, the calculated value of the standard error would have been 18.1%. Clearly, if there is as much variation of $\delta^{15}N$ values across the site as in this example, it is necessary to determine the correlation between $\delta^{15}N$ values of reference and N_2-fixing plants, if the site is to be of use for the natural-abundance method of estimating N_2 fixation.

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5

Nitrogen Fixation in Soil and Plant Systems

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I. INTRODUCTION

Since the last century, when evidence accumulated indicating that microorganisms and nodulated legumes were capable of utilizing atmospheric nitrogen, progress in the field of N_2 fixation was almost entirely dependent on the development of suitable methods and experimental techniques of measurement. Three major technological steps facilitated studies of N_2 fixation: the development of reliable methods for estimating the nitrogen content in organic material; the discovery and use of N isotopes as tracers; and the development of other simple, sensitive, indirect methods such as that employing acetylene reduction. Frequently, the advent of one new method momentarily overshadowed the previous one. Interestingly enough, however, the most useful and commonly used methods for estimating N_2 fixation today can still be classified into three groups based on these three main developments: nitrogen accumulation, acetylene reduction, and use of ^{15}N. Each has its advantages and limitations.

A. Nitrogen Accumulation Method

Nitrogen accumulation is the oldest and most conventional method for estimating the rate of nitrogen fixation. The increase over time of the total N in all compartments of a system (soil, vegetation) or differences in N accumulation between N_2-fixing and nonfixing plants are traditionally determined by the Kjeldahl method. Measurements are made over

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substantial periods of time so that changes in N content can be distinguished from the measurement error. Adequate for systems relying solely on atmospheric N, this method has limited application in more complex systems such as plant–soil systems. Overall nitrogen balance, or estimating the gain of N from biological fixation in ecosystem studies, is difficult because of the many possible N inputs and outputs and the various degrees of precision in measuring them.

B. Acetylene Reduction Method

Acetylene reduction is based on the ability of nitrogenase to reduce substrates other than N_2, principally acetylene to ethylene, which can be easily measured by gas chromatography. Rapid, inexpensive, and extremely sensitive, this method continues to be intensively used for detecting N_2 fixation capacity and for making comparative studies of N_2-fixing systems. However, it suffers from serious drawbacks because (1) it can only be of short duration (due to diurnal and seasonal variation in N_2 fixation, the acetylene reduction assay is difficult to extrapolate over a long period of time), and (2) the theoretical conversion ratio of 3 used to convert amount of ethylene produced to amount of N_2 fixed has been found to vary widely among plant and environmental conditions. Burris (1974) has exhorted users to establish valid conversion factors for assessment of N_2 fixation and stressed that reduction of $^{15}N_2$ is the primary method for validation. Moreover, the acetylene reduction method is not very well suited for field studies. Nevertheless, it remains very useful for qualitative studies of N_2 fixation and for testing biological material before undertaking more tedious and expensive methods such as those involving $^{15}N_2$.

C. Isotope Methods

As often emphasized in this series of volumes , tracing of one element in complex biological systems can be most accurately achieved if several isotopes of this element exist and are available for experimentation. There are several known isotopes of nitrogen, but the most easily detectable ones, radioisotopes, are not suitable for research in biological systems because even the longest-lived among them, ^{13}N, has a half-life of only 10 min (see Chapter 10). Although it has been used in N_2 fixation studies, the technical difficulties involved do not allow its general use.

Stable isotopes of nitrogen ^{14}N and ^{15}N naturally occur at a ratio of 272 : 1 or 0.3663 atom% ^{15}N in atmospheric N. However, there are differences between atmosphere and soil. By using these differences (natural ^{15}N abundance method) or by artificially inducing them through the addition of ^{15}N, either in the atmosphere ($^{15}N_2$ method) or in the soil (^{15}N–enriched fertilizer method, often called the ^{15}N isotope dilution method), it is possible to estimate the amount of N that is derived from biological N_2 fixation. Based on the classical concept of isotope dilution, these various methods have advantages and limitations that make them suitable only in certain conditions. This chapter will therefore be devoted largely to the use of ^{15}N–enriched atmosphere and soil methods, the natural ^{15}N–abundance method being dealt with in Chapter 4.

II. $^{15}N_2$ REDUCTION METHOD

A. Principles and Theory

Soon after ^{15}N became available, $^{15}N_2$ was used to provide evidence for N_2 fixation (Burris and Miller, 1941). Overall, the use of $^{15}N_2$ as a tracer is the most satisfactory method in the field of N_2 fixation. Somewhat superceded by the acetylene reduction technique for the economical and technical reasons mentioned, $^{15}N_2$ use is now regaining interest due to the recent developments in ^{15}N tracer techniques. Despite difficulties in its use, the $^{15}N_2$ incubation method remains the absolute method of measurement of N_2 fixation against which other methods should be tested. Moreover, many qualitative and quantitative aspects of translocation and fate of biologically fixed N can be investigated only through the use of $^{15}N_2$.

The principle of $^{15}N_2$ incorporation is simple. The whole fixing system (bacterial preparation, soil, plant, or root) is exposed to an atmosphere enriched in $^{15}N_2$ for a specified period of time followed by ^{15}N determination in the material exposed. The whole system or its separate components may be analyzed. The percentage of total N that was fixed (%Ndfa) is determined by the equation

$$\%\text{Ndfa} = \frac{^{15}\text{N atom\% excess in sample}}{^{15}\text{N atom\% excess in atmosphere}} \times 100. \qquad (1)$$

If the total amount of N in the sample is known, it is possible to calculate the real amount of N fixed during the exposure period.

B. Materials Required

1. Source of $^{15}N_2$

Nitrogen gas enriched in ^{15}N can now be purchased conveniently from commercial sources in 0.5- and 1-liter light, alloy bottles provided with a tap (e.g., ORI, Gif sur Yvette, France). Enrichments of 50, 95, and 99% are generally available. Because of the high cost of gaseous ^{15}N and the difficulties in shipping and handling, production from labeled ammonium salts may be favored. Besides being economically more convenient, this is suitable when small amounts of $^{15}N_2$ are required. Prepared immediately before use with biological material, it avoids problems associated with storage, such as contamination with atmospheric N_2 and consequent decline in the ^{15}N content. Ammonium salts can be readily converted to $^{15}N_2$ gas by oxidation. Oxidation is best obtained by passing labeled ammonia over hot copper oxide after generation with sodium hydroxide. This is achieved in an evacuated apparatus similar to that described by Bergersen (1980). A simpler way to generate $^{15}N_2$ is by oxidation of ammonium salts with alkaline hypobromite, although it produces more impurities. In all cases, whether purchased or produced locally, $^{15}N_2$ must be purified because traces of nitrogen oxides and ammonia may affect the measurement of nitrogen fixation. Impurities are removed by passing $^{15}N_2$ gas through a liquid air trap or solutions of $KMnO_4$-KOH and H_2SO_4. The following method is one of the simplest ways to generate and purify $^{15}N_2$. It is adapted from Ohyama and Kumazawa (1981).

2. Preparation and Purification of $^{15}N_2$

a. Reagents Hypobromite iodine solution is obtained by slowly adding 120 ml of Br_2 to 600 ml of 16 *N* NaOH solution continuously stirred and maintained below 5°C. After 1 wk in a refrigerator, the solution is filtered to remove the precipitate and the supernatant is mixed with an equal volume of KI solution (0.2% w/v). One milliliter of this hypobromite iodine solution will oxidize 5–6 mg of NH_4^+-N to N_2.

$KMnO_4$-KOH is obtained by dissolving 5 g $KMnO_4$ and 2.5 g KOH in 100 ml distilled water.

Na_2SO_4-H_2SO_4 is prepared by dissolving 20 g Na_2SO_4 in 100 ml H_2SO_4 (5% v/v).

$(^{15}NH_4)_2SO_4$ with 99% enrichment is commercially available.

b. Procedure Preparation, purification, and storage of $^{15}N_2$ can be achieved with an apparatus such as that described in Fig. 1A. Each piece of glassware is connected by pressure tubing. The stopcocks are either greased glass or a high-vacuum greaseless type and the flasks may be round-bottomed standard taper ground joint flasks. The volume of each flask is adjusted according to the total volumes of the reagents and of the $^{15}N_2$ gas at 1 atm.

When all the reagents have been placed in the appropriate flasks, the whole system is evacuated to about 0.1–1.0 Pa (see Chapter 3, Section II.B, for interconversion of units) with a vacuum pump connected at point a. The vacuum is maintained for 1 hr to ensure the complete outgassing of the system. $^{15}N_2$ gas is then generated by drop-wise addition of hypobromite iodine solution to the ammonium salt, stopcock 1 being closed. Slightly acidified distilled water is then added to remove the excess vacuum. $^{15}N_2$ gas is displaced to the next flask by slowly opening stopcock 1, stopcock 2 being open and 3 closed. After water has completely replaced the gas in the generation flask, stopcock 1 is closed. The same procedure is followed to displace $^{15}N_2$ to the second purification flask using the appropriate stopcocks and adding water from b and then c. The purified gas is then transferred to a storage bottle previously filled with acidified distilled water to trap any dissolved NH_3 from laboratory air. Drainage water is collected

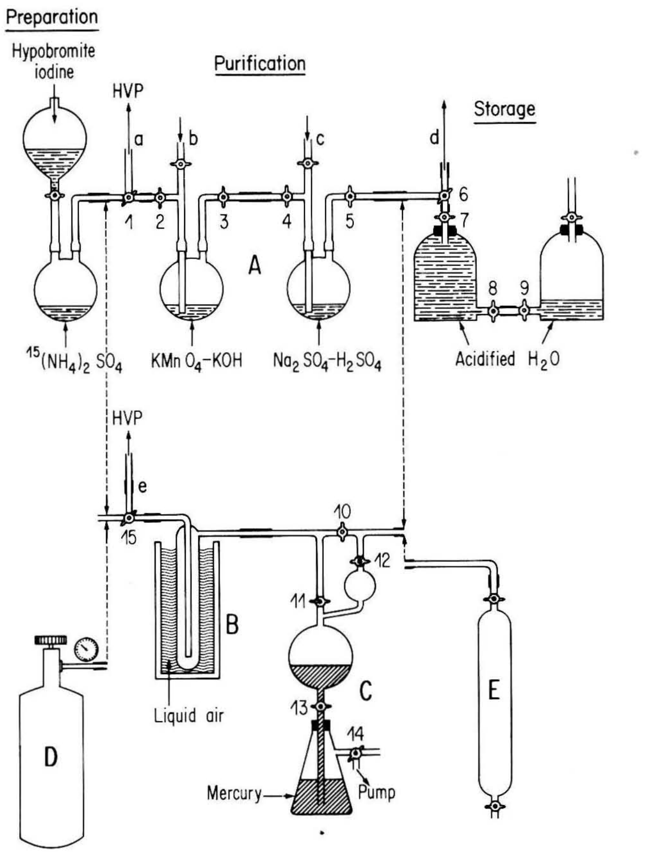

Figure 1 Apparatus for preparation, purification, and storage of $^{15}N_2$. (A) Preparation from ammonium salt, purification with $KMnO_4$-KOH and Na_2SO_4-H_2SO_4, and storage in bottles. (B) Purification with cold trap. (C) Toepler pump. (D) Commercial preparation. (E) Storage in glass ampoule. 1, 2, ..., stopcocks; HVP, high-vacuum pump; a, b, ..., openings. Thick lines represent vacuum tubing. For more information, see text.

in another bottle containing water to prevent back flow of air from stopcock 8. $^{15}N_2$ gas can then be used and taken directly from stopcock 7 after the bottle has been separated from the system. This is done by the addition of water through stopcock 8. The bottle to the right of stopcock 8 could be replaced by a separatory funnel connected by a flexible hose.

*c. **Alternative*** Purification of locally prepared or purchased $^{15}N_2$ may also be achieved by passing the gas through a cold trap refrigerated with liquid N_2 or liquid air, as pictured in Fig. 1B. In this case, water cannot be used for gas displacement, which is instead easily achieved with a Toepler pump, as pictured in Fig. 1C. The complete procedure is as follows. The whole system is connected, including the source of $^{15}N_2$ [either the generation flask (as in Fig. 1A) or a commercial gas tank (Fig. 1D)], the purification train, the Toepler pump (Fig. 1C) and the storage container [bottle as in Fig. 1A or glass ampoule provided with stopcocks (Fig. 1E)]. High vacuum (0.1–1.0 Pa) is applied from e in Fig. 1B,C,E, stopcocks 11 and 12 being closed and the mercury in the Toepler pump being raised up 11 and 12. $^{15}N_2$ is then introduced into the system through stopcock 15, stopcock 10 being closed. Circulation of the gas from Fig. 1B to E is achieved by the Toepler pump. With stopcocks 10 and 12 closed and 11 opened, the mercury is lowered to stopcock 13 by connecting 11 to a vacuum pump. Stopcock 11 is then closed, 12 is opened, and the mercury is raised to 12 by connecting 14 to the atmosphere. This allows the gas to be pumped to Fig. 1E. The same operation is repeated several times to collect the maximum amount of $^{15}N_2$.

3. Biological Materials

Although the ability to fix nitrogen is limited to a small number of organisms, bacteria including cyanobacteria, they are widespread in nature, often in association with other organisms. Almost every kind of material suspected to harbor nitrogen fixers has been tested for nitrogen fixation. As long as it can be enclosed in a rather small volume, the biological material can be assayed with $^{15}N_2$. However, in order of increasing technical difficulties, material can be classified as follows:

- Material that can be enclosed completely in small-volume containers [e.g., preparations of free-living microorganisms, soil samples, plant parts (leaves, roots, nodules)].
- Material that requires larger containers for partial enclosure (e.g., higher plants with or without their soil, for which only the belowground part is exposed to $^{15}N_2$: symbiotic systems, such as symbioses with legumes or nonlegumes or nonsymbiotic systems, such as grasses with their rhizosphere).

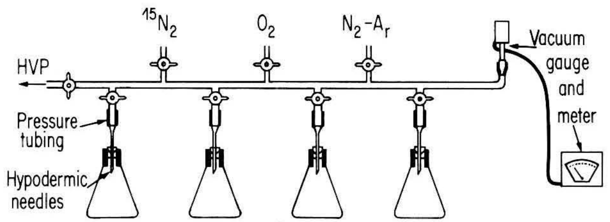

Figure 2 Apparatus for exposure of samples to artificial atmosphere containing $^{15}N_2$, including Erlenmeyer incubation flasks connected to a manifold for preparation of gas mixtures and a vacuum measuring device. HVP, high vacuum pump.

- Higher plants that require complete enclosure in a large container due to internal connections between roots and atmosphere (e.g., rice or other flooded plants as well as some species in the Graminaceae with aerenchyma).

A distinction also has to be made between aerobic and anaerobic organisms, which require different incubation conditions.

4. Incubation Systems

Various types of enclosures have been used in $^{15}N_2$ experiments according to the type of biological material to be tested for nitrogen fixation or related processes. However, there are general features: (1) the incubation system must be perfectly closed and impermeable to gases in order to maintain a constant $^{15}N_2$ gas phase, (2) the volume must be small to reduce the amount of $^{15}N_2$ needed and to increase the sensitivity of measurement at a reasonable cost, and (3) the volume must be sufficient to ensure ample supply of gases such as oxygen in the case of aerobic N_2 fixation and provide sufficient dilution of accumulating gases such as CO_2, which may affect biological processes. In some cases, these parameters must be regulated, hence increasing the technical difficulties.

*a. **Isolated components*** When the biological material can be completely enclosed (as is the case for microorganisms, soil samples or cores, and plant parts or tissues), almost any kind of vessel can be used providing that it is made of a material that is impermeable to N_2 and suitable for use with a gas-sampling device for introduction and removal of $^{15}N_2$ or other gases. Glass vessels such as Erlenmeyer or Warburg flasks are very convenient when vacuum must be made to prepare artificial gas mixtures. They must provide an adequate opening for connection to a gas manifold (Fig. 2) or for serum stopper or Suba seal for injection and removal of gases with a

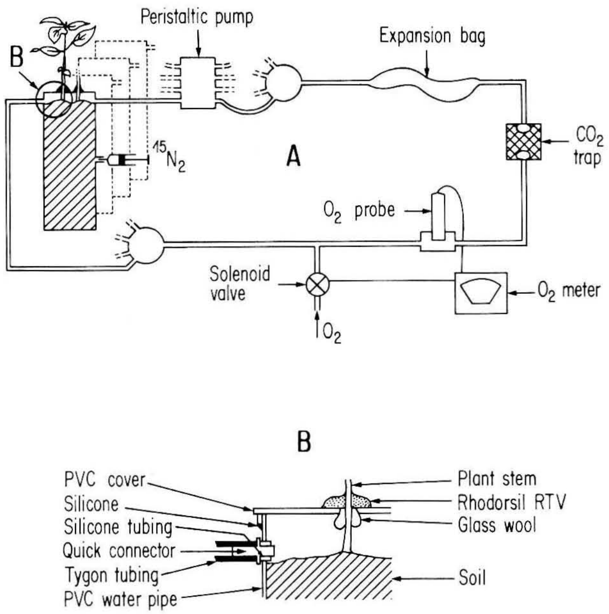

Figure 3 Equipment and apparatus for $^{15}N_2$ exposure of plant–soil systems. (A) Closed-circuit system including O_2 regulating device. (B) Detail of plant container indicating sealing material for plant stems and aerations ports.

syringe. Plastic bags can also be used and have the advantages of being adaptable to almost any size of biological material and of being collapsable, which provides a better evacuation than with glass flasks, and a volume under the complete control of the investigator. However, the choice of a suitable plastic is important because the rates of gas diffusion through plastics are markedly different. Saran material is the least permeable to gases (Burris, 1974).

b. Higher plants and plant–soil systems For studies involving entire symbiotic systems such as legumes or nonlegume plants growing in nutrient solution, in sand or in soil, special containers designed to house the soil and to allow enclosure of the soil atmosphere around the base of the plant stem are necessary. As indicated in Fig. 3, this can be achieved using PVC water pipes provided with a bottom and a cover glued with silicone rubber. Holes drilled through the cover allow emergence of the stems. Holes must be tightly sealed before an experiment, using physiological molding material such as Terostat (Teroson, Heidelberg, Germany) or RTV silicone rubber. For the latter, a layer of glass wool must be placed around the stem in the hole in order to hold the sealant, which has a liquid consistency before vulcanization by a catalyst. Several openings should be provided for aeration, drainage of water, and sampling. They may be connected in a closed-circuit system, plugged with rubber stoppers, or fitted with serum caps during the exposure to $^{15}N_2$. One method to make tight connections through the PVC walls of the containers is to drill holes of a diameter slightly larger than that of a plastic quick connector and then to insert a piece of silicone tubing. The connector can then be forced into the tube and through the wall to provide a perfect airtight connection to which tygon or silicone tubing can be adapted (Fig. 3B). The size of the container may be easily chosen according to the plant under investigation because PVC pipes are available in various sizes.

Nonsymbiotic plants may also be exposed to $^{15}N_2$ with their soil in the same type of containers. Due to the small nitrogen fixation activity likely to occur in their rhizosphere, exposure must be maintained for longer periods of time. This requires control of atmospheric parameters and therefore increases the possibility of leakage.

In the case of rice and other plants that grow in water-logged soils, gases diffuse from the shoot through the expanded cortical aerenchyma. For this reason, it is necessary to enclose the whole plant for $^{15}N_2$ exposure. Control of environmental parameters such as light, CO_2, and water vapor become essential to maintain photosynthesis. In addition, the volume of air to be enriched by $^{15}N_2$ is larger than that when only the root system is exposed. Controlled chambers consisting of a photosynthesis canopy and a root container have been designed for this purpose. Their construction is based on techniques developed by plant physiologists for measurement of photosynthesis or exposure to $^{14}CO_2$. Descriptions of systems with automatic control of temperature, CO_2 concentration, and water vapor are given by Warembourg and Kummerow (1991) in Volume 2, Chapter 2, of this series. Adaptation for $^{15}N_2$ studies requires reduction of the aerial part of the chamber so that the $^{15}N_2$ cost does not become prohibitive.

5. Environmental Control

For quantitative estimation of biological N_2 fixation, especially for long-term exposures, environmental parameters that affect the fixation process and the activity of the material as a whole should be carefully maintained within nonlimiting ranges. This includes temperature, O_2 and CO_2 concentration, and, in some cases, N_2 concentration.

a. Temperature If necessary, temperature can generally be adequately controlled using thermoregulated water baths in which the $^{15}N_2$

exposure vessels can be immersed. An exception is when the entire plant is enclosed and exposed to light. In this case, temperature control requires more complex equipment such as that described by Warembourg and Kummerow (Volume 2, Chapter 2). These cases will not be emphasized in this chapter.

b. Concentration of O_2, CO_2, and N_2 in the gas-phase In the case of short-term exposure experiments, no regulation of these parameters is necessary. However, because isotopic N is expensive, the need to reduce the volume of gas may require some precautions. With aerobic N_2-fixing systems, changes of O_2 concentration may influence the activity. The gas volume must therefore be large enough to avoid significant reduction in O_2 due to respiration. The same applies for CO_2, which may also affect N_2-fixing activity. The concentration of N_2, although not limiting in normal air, may become limiting if artificial atmospheres with low pN_2 values that are diluted with an inert gas such as helium or argon are utilized to reduce the amount of $^{15}N_2$. Although this procedure is acceptable for comparative purposes, quantitative estimations of N_2 fixation must be made in atmospheres containing 78% N_2.

In the case of plant and plant–soil experiments, when the root system is enclosed for periods of several hours to several days, O_2 concentration quickly becomes limiting and a regulation system allowing addition of O_2 is highly desirable. As illustrated in Fig. 3, this is achieved using a closed–circuit system including the root incubation vessel, a peristaltic pump fitted with silicone tubing, an expansion bag, a CO_2 trap, and an oxygen measurement device. The bag (medical disposable urine bag) is designed to compensate pressure changes during introducion of $^{15}N_2$ and O_2. Oxygen is measured using a polarographic probe (Clark type) suitable for gas-phase measurement and connected to an oxygen meter.

The soil atmosphere is continuously circulated by the pump. When necessary, the oxygen meter operates a solenoid valve connected to an oxygen tank, thus maintaining O_2 concentration at a preset value. Effluent CO_2 is absorbed on soda lime in the CO_2 trap.

6. Sampling

Because $^{15}N_2$ techniques are not used in field situations, there is no need for special sampling equipment, only facilities for handling, sampling, drying, and milling samples of soil or vegetation. For gases, disposable plastic syringes and needles are convenient. Samples may be stored in prevacuated tubes (vacutainers), in which case leakage is reduced by storage under water.

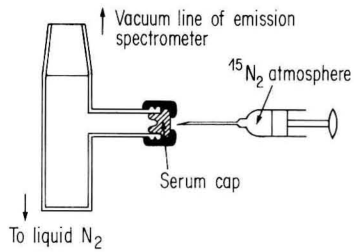

Figure 4 Apparatus for introduction of atmosphere samples enriched in $^{15}N_2$ into emission or mass spectrometer.

7. Analysis

As indicated in Chapters 2 and 3, all methods of ^{15}N measurement involve the conversion of nitrogen in a sample to N_2 gas, which is isotopically analyzed by emission or mass spectrometry.

a. Gas samples Calculation of N_2 fixation using $^{15}N_2$ requires knowing the ^{15}N concentration in the gas used to expose the N_2-fixing system under study. This can be estimated from the enrichment given with the source of $^{15}N_2$ by correcting for dilution with N_2 sources, using the volume of the exposure system. However, it is strongly recommended that direct measurement of gas samples be made during the course of the experiment. This is also the only way to detect possible leakage from the system. This measurement can be performed by emission or mass spectrometry. In both cases, an adaptation device such as that illustrated in Fig. 4 is connected to the vacuum line of the apparatus to allow direct injection of gas samples to be analyzed for ^{15}N.

b. Soil and plant samples Analysis of ^{15}N concentration of solid material involves the conversion of nitrogen compounds into ammonium and then the conversion of ammonium into $^{15}N_2$ in the sample preparation line of the emission or mass spectrometer (see Chapters 2 and 3). The first conversion is commonly done with the Kjeldahl method, in which total nitrogen is converted to NH_3 in hot concentrated sulfuric acid in the presence of a catalyst (Wieninger reagent). The NH_3 is recovered by steam distillation, and total N is estimated by titration.

Material is digested in glass tubes held vertically in a heated steel block and ammonia is recovered by distillation. Excess acid is titrated with NaOH on a pH titrimeter to avoid use of a color reagent, which could interfere with the measurement of ^{15}N. Samples are then adjusted to a pH of 3–4 and evaporated by heating at 60°C. Dehydration is completed in a desiccator in the presence of phosphoric anhydride. The resulting solid ammonium sulfate can be stored under vacuum until analysis in a spectrometer. Other details and methods are described in Chapters 2 and 3.

C. Description of Procedures

Use of $^{15}N_2$ is mainly recommended for two research objectives: (1) to give an absolute value for N_2 fixation, by itself or in relation to related processes such as carbohydrate utilization or CO_2 fixation by legume nodules, and (2) to follow the fate of fixed N within different components of the fixing system (e.g., plant parts) or biochemical components. Whatever the objective, ^{15}N is incorporated through biological fixation, by exposing the fixing system to an atmosphere containing $^{15}N_2$. The atmosphere of the entire system can be replaced by an artificial gas mixture containing $^{15}N_2$, or the normal atmosphere can be labeled *in situ* with $^{15}N_2$. Once ^{15}N has been incorporated, the sampling procedure can be adapted to measure N_2 fixation or to investigate translocation of nitrogenous compounds.

1. Level of ^{15}N Enrichment

A certain amount of information is needed before determining the level of ^{15}N enrichment to be used for $^{15}N_2$ exposure of N_2-fixing systems. First, the type of spectrometer available for ^{15}N determination may limit sample size and minimum detectable enrichment. Modern mass spectrometers fitted with a dual injection system require a sample of 250 μg N for a detection limit of 0.0004 atom% excess. An emission spectrometer has considerably lower sensitivity, 0.010 ^{15}N atom% excess, but samples of 6–10 μg are suitable for routine analysis. Second, an approximation of the extent of N_2 fixation to be expected during the experimental period should be made. In many cases, this can be estimated from acetylene reduction assays (for details, see Turner and Gibson, 1980). A correct minimum estimate will help ensure that at least some ^{15}N will be detected. Third, the nitrogen content of the experimental material into which fixed N is diluted must be known. From this and the sensitivity of the ^{15}N measurement, the sample size and number of replicates can be chosen.

An example will illustrate the type of calculations required before a $^{15}N_2$ exposure experiment. Let us suppose that the rate of N_2 fixation is to be measured at different periods during the growth cycle of soybean plants and that the method chosen is a 1-day exposure of the root system to $^{15}N_2$. A mass spectrometer is available for ^{15}N determinations.

The total dry weight of soybean plants grown outside varies from 5 g, 50 days after sowing, to >100 g, at maturity (day 150). Assuming a mean plant nitrogen concentration of 1.5%, the N per plant ranges from 75 to

1500 mg. Given variations in biological material, adequate measurement of ^{15}N should be at least 10 times the detection limit of the mass spectrometer, which is 0.0004 atom% excess. The minimum fixation rate should give this average enrichment for the whole plant or includes 0.004% × 75 to 0.004% × 1500 mg ^{15}N in excess of natural, i.e., 0.003–0.06 mg ^{15}N in excess. Soybean crops have been reported to fix between 0.1 and 1 g N/plant in 100 days or an average of 1–10 mg/day. The minimum enrichment to meet the requirements of all experiments should therefore be $0.06/1 \times 100 = 6\%$ ^{15}N atom in excess. Enrichments as low as $0.003/1 \times 100 = 0.3\%$ ^{15}N atom in excess may be sufficient when the plants have a low biomass. However, because fixed N is not evenly distributed into all plant parts, safety limits for ^{15}N measurements require higher enrichments. If the volume of the gas phase in the experimental exposure system, including the plant containers and the connections, is 1 liter/plant, and if a natural atmosphere is used, then this atmosphere should contain $780 \times 6\% = 46.8$ ml pure $^{15}N_2$ per plant. This can be achieved by addition of the equivalent using a lower enriched source of $^{15}N_2$ (e.g., about 100 ml of gas containing 50 atom% ^{15}N).

2. Artificial Atmospheres for Short-Term Exposure

This procedure is chosen for isolated components such as bacterial preparations, soil samples, and separated plant parts. It is highly desirable for anaerobic fixing systems. A suitable system is illustrated in Fig. 2. Samples are transferred into exposure vessels, which are closed using the appropriate stoppers. Fresh weights can be determined before introduction but the exact weight of the samples may be more conveniently determined after exposure by oven drying. Vessels are connected to the manifold and the appropriate atmosphere established as follows. The system is completely evacuated with a high vacuum pump by opening the appropriate stopcocks, the vacuum level being controlled with a vacuum gauge and meter. The appropriate amount of ^{15}N-enriched nitrogen is then injected into the system from a syringe or directly from a storage container (Fig. 1). This is done by slightly opening the stopcock and carefully controlling the vacuum meter until the required amount is taken in. If required, O_2 is then introduced following the same procedure, and the whole system is brought to atmospheric pressure by backfilling with Ar or He. Incubation follows under the desired conditions of temperature and light, often using a water bath, for the preset period of time. The gas phase should be sampled at appropriate intervals to monitor the ^{15}N atom% of the experimental atmosphere. At the end of incubation, the system is washed of the remaining $^{15}N_2$ by several evacuations, refilling each time with the normal atmosphere or with an inert gas such as Ar or He. Sampling of material and preparation for ^{15}N analysis can then follow.

Comment: A savings of $^{15}N_2$ and an increase in sensitivity of measurement can be achieved by choosing a N_2 partial pressure lower than that of normal air. However, a pressure of at least 40 kPa is necessary to saturate the N_2-fixing sites.

It is necessary to include a control that is not exposed to $^{15}N_2$ but otherwise treated in exactly the same way as the exposed sample, so that natural ^{15}N abundance among replicates can be corrected for. This is particularly important when working with short periods of time, as is often the case with this method, and with biological material of low N_2-fixing activity.

3. Labeling the Root Atmosphere

In the case of whole plant experiments, where the belowground part is exposed to $^{15}N_2$, care must be taken that severe pressure changes do not induce leakage around the soft and elastic material used to close the system around the plant stems, especially during complete replacement of a normal atmosphere. Moreover, with the volume being generally large, the complete removal of N_2 is difficult. For these reasons, the recommended method is to label the normal atmosphere with an appropriate amount of $^{15}N_2$. Suitable for plant–soil systems of rather large volumes, this method is most adequately used with an environmental control, thus allowing long-term exposure. The procedure will be described for a complex system involving legume plants grown in soil. The same description applies for less complex systems such as plants grown in sand or liquid nutrient solutions or for nonsymbiotic systems such as plant and rhizospheric N_2-fixing associations, which however require a longer exposure to $^{15}N_2$ due to low N_2 fixation rates. The equipment used is that illustrated in Fig. 3 and described in Section II.B.4.

The plants may be directly grown in the containers designated for exposure to $^{15}N_2$ or transferred before experiments. Removal from the plant bases of any dying material is important to prevent leakage when applying the sealing material. Using RTV silastic, a period of several hours is needed for complete vulcanization. Checking for leaks is easily done by immersing the containers into water, the various openings being closed with rubber stoppers except one that is used to raise the pressure by slow introduction of air. This is done by blowing gently, the occurrence of leaks being indicated by bubbling. Leaks are corrected by adding more sealing material. The openings of the containers are then closed or connected to the pump. After the air inlet is opened, the pump is activated for several minutes to renew the inside air and to purge the system of excess CO_2 that may have accumulated during preparation. The entire system is then connected and placed in a location suitable for the experiment: outside, in a greenhouse, or in a lighted cabinet.

The amount of $^{15}N_2$-enriched gas calculated as meeting the requirements of the experiment (see earlier) is then introduced into the system. It may either be injected into each container with a syringe (as indicated in the figure) or supplied from a gas ampoule (such as that illustrated in Fig. 1E) directly inserted into the system. The peristaltic pump then circulates the gas into each container and out into the common circuit to thoroughly mix the inside atmosphere. Increase of volume due to injection of $^{15}N_2$ is compensated by the expansion bag; decrease in the relative O_2 concentration is adjusted for by the O_2-regulating device.

The exposure period for legumes is often set to the photoperiod. In this case, the $^{15}N_2$ atmosphere is maintained during 24 hr (one photoperiod and the following night). However, shorter or longer exposure periods may be chosen according to the specific experiment. Throughout the exposure period, the ^{15}N enrichment of the experimental atmosphere is determined on soil atmosphere samples taken at regular intervals. These samples (2 or 3 ml) are taken with disposable syringes and immediately stored into evacuated tubes (Vacutainers). ^{15}N content is most easily determined using emission spectrometry. Mass spectrometry may also be used if available.

At the end of the exposure period, each container is opened and flushed with normal air for 1 hr to remove the residual $^{15}N_2$. Sampling of plant material may proceed after removal of all connections and sealing material.

Comment: When working with soil, at least 1 hr is needed to evenly distribute N_2 in the exposure system (Fernandez and Warembourg, 1983). However, the size of the system as well as soil characteristics such as texture and moisture may slightly change the diffusion pattern of $^{15}N_2$. This will show up in the time course of ^{15}N enrichment, as measured by periodic sampling of soil atmosphere. The time delay is of utmost importance in fixing the length of the exposure period because during this delay N_2 fixation sites are not exposed to maximum ^{15}N enrichment. In such conditions, the exposure period should exceed several hours.

As indicated, plants not exposed to $^{15}N_2$ but grown in the same conditions are used as controls for natural abundance in ^{15}N.

4. Sampling and Analysis of the Biological Material

For estimating N_2 fixation, plant material can be sampled immediately after the $^{15}N_2$ exposure period. However, for studying distribution of fixed N among plant parts, the first sampling can only be done after the nitrogen compounds have been translocated from the site of fixation (e.g., nodules) to the various sinks. A period of 48–72 hr after ^{15}N incorporation has been found to be the lower limit for most legume plants to get a stable distribution.

Measurement of N_2-fixation rate requires only the determination of the total amount of ^{15}N incorporated into the biological material during exposure to $^{15}N_2$. However, due to variations in incorporation and especially for large samples such as plant material, it may be necessary to separate samples into categories such as belowground and aboveground organs and soil.

Measurement of fixed-N distribution and fate always requires a careful separation into components such as nodules, roots, stems, leaves, reproductive structures, and soil.

This is easily done for the aerial portion of the plants using scissors or scalpels. The belowground parts such as roots and nodules must be washed clean of soil particles. This is most commonly done by washing over sieves of several mesh sizes to retain all the roots.

When periodical sampling must be conducted after exposure to $^{15}N_2$, enough plants not exposed to $^{15}N_2$ must be available for determination of natural abundance, which is known to change according to the source of N (soil or atmosphere). This change mainly occurs in legumes but must be taken into account for nonlegumes with low rates of N_2 fixation.

Samples are placed in paper bags and oven-dried at 70°C. They can then be stored until N and ^{15}N analysis are undertaken.

Analysis follows the procedures described in Chapters 2 and 3. Care should be taken to proceed from the less enriched material to the more enriched to prevent contamination, even when equipment is washed according to recommendations.

A set of glassware and apparatus must be kept free of ^{15}N-enriched material for analysis of the controls not exposed to $^{15}N_2$.

5. Calculations

The following values are measured:

1. Dry matter in different plant parts (e.g., seeds, pods, leaves, stems, crowns, roots, nodules),
2. % of N in each plant part,
3. ^{15}N atom% of each plant part separately for plants exposed and not exposed to $^{15}N_2$, and
4. ^{15}N atom% in the experimental atmosphere.

Calculations can be made as follows. ^{15}N atom% excess in plant parts is figured from

$$\mathrm{AE}_{(a)} = \mathrm{A\%}_{(a)}(\text{plant exposed to } ^{15}\mathrm{N}_2) - \mathrm{A\%}_{(a)}(\text{plant not exposed}) \quad (2)$$

where $\mathrm{AE}_{(a)}$ is ^{15}N atom% excess in part a, and $\mathrm{A\%}_{(a)}$ is ^{15}N atom% in part a.

According to the general Eq. (1), percentage of N derived from atmosphere, or %Ndfa, into a plant part is

$$\%\mathrm{Ndfa} = \frac{\mathrm{AE}_{(a)}}{\mathrm{AE\ atmosphere}} \times 100 \quad (3)$$

A weighted atom% excess for the whole plant can be calculated as follows:

$$\mathrm{WAE} = \frac{\mathrm{AE}_{(a)} \times \mathrm{TN}_{(a)} + \mathrm{AE}_{(b)} \times \mathrm{TN}_{(b)} + \ldots \mathrm{AE}_{(n)} \times \mathrm{TN}_{(n)}}{\mathrm{TN}_{(a+b+\ldots n)}} \quad (4)$$

where WAE is weighted ^{15}N atom% excess, and TN is total nitrogen in plant parts a, b, ..., n and total N_2 fixation per plant and per unit of time is

$$\mathrm{N_2\ fixed\ pl^{-1}\,hr^{-1}} = \frac{\mathrm{WAE} \times \mathrm{TN}_{(pl)}}{\mathrm{AE\ atmosphere} \times t} \quad (5)$$

where $\mathrm{TN}_{(pl)}$ is total N per plant, and t is time of exposure in hours.

Distribution of fixed N can be calculated for each plant part using the following:

$$\mathrm{fixed\ N}_{(a)} = \frac{\%\mathrm{Ndfa}_{(a)} \times \mathrm{TN}_{(a)}}{\mathrm{N_2\ fixed\ pl^{-1}}} \times 100 \quad (6)$$

The fate of fixed N as a function of time after $^{15}N_2$ exposure is calculated from the differences in ^{15}N content of plant parts. If volatilization or exudation of N compounds do not occur, as is generally the case, and if all plant parts are collected, the total amount of ^{15}N does not change but, rather only its distribution among different plant parts.

D. Comments

The main limitation of the $^{15}N_2$ reduction method is that it is technically difficult. Because the fixing system must be exposed to an $^{15}N_2$ atmosphere of constant enrichment, sophisticated and expensive apparatus are required to prevent leaks and maintain normal environmental conditions. Even with maximum precaution, leakage can occur, especially when working with higher plants. This can be monitored by measuring the ^{15}N content of the gas phase in the closed system so that N_2 fixation can be calculated based on an average value of ^{15}N concentration during the assay. However, the equipment cannot accommodate many replicates and is not suitable for routine field measurements. In addition, the method is destructive, complicating repetitive sampling over long-term experiments. Fortunately, it has been found that plants growing in separate containers show less variability in N_2-fixation rates and related growth processes than field-grown plants, so that fewer replicates are needed.

The $^{15}N_2$-reduction method remains a short-term kinetic measurement and, as such, is not useful for integrated quantification of N_2 fixation. However, it represents a powerful tool in fundamental research because it is the only direct method of estimating N_2 fixation. The isotopic element is incorporated by biological processes and, hence, behaves as a true tracer of fixed N. This allows important applications. As emphasized, the $^{15}N_2$ reduction method is the primary method used to evaluate the accuracy of results obtained by acetylene and other indirect methods for measuring N_2 fixation. It is the only absolute measurement that demonstrates the occurrence of N_2 fixation and can screen bacterial strains and associations between plant cultivars and bacteria for N_2-fixation efficiency.

Use of $^{15}N_2$ is also the method of choice for investigation of translocation patterns of fixed N into N_2-fixing systems. When associated with studies involving other processes related to N_2 fixation, $^{15}N_2$ reduction is the only way to obtain quantitative relationships. Simultaneous labeling of plants with $^{15}N_2$ and $^{14}CO_2$ has proven very useful in the study of carbon and nitrogen economy in N_2-fixing plants (Warembourg *et al.*, 1982). The method based on short-term exposure of aerial parts of plants to $^{14}CO_2$ and belowground parts to $^{15}N_2$ is meant to study carbon use in relation to N_2 fixation. Respiration studies of nodulated roots associated with N_2 reduction permit estimation of the carbon costs of nitrogen fixation. Other processes associated with nitrogenase activity, such as PEP carboxylase activities in nodules, can also be quantified using double labeling. In this case, only the root atmosphere is labeled with $^{14}CO_2$ and $^{15}N_2$, and the amount of isotopes recovered in the plants is a reflection of the magnitude of each process.

In conclusion, the $^{15}N_2$ reduction method, the oldest ^{15}N isotope-aided method applied to N_2 fixation, has had relative successes since its first application by Burris and Miller (1941). More progress is expected, due principally to improvement of the equipment used for ^{15}N determination and environmental control of plant growth. Technical difficulties and costs no longer prohibit a more general adoption of the $^{15}N_2$ reduction method.

III. ^{15}N ISOTOPE DILUTION METHOD

A. Principle and Theory

The basic principle of the isotope dilution method, as applied to N_2 fixation studies, is similar to that of the $^{15}N_2$ exposure method. The only difference is that the soil is enriched in ^{15}N instead of the atmosphere. By adding ^{15}N-enriched fertilizers to soil, one ensures that the plant will take up N from soil with a higher ^{15}N content than that in the atmosphere. The extent to which this ^{15}N enrichment is diluted by atmospheric N in a fixing plant reflects the magnitude of fixation. Accordingly, estimation of the amount of N_2 fixed requires only the measurement of (1) the ^{15}N abundance of the N_2-fixing plant, (2) the ^{15}N abundance of the soil N, and (3) the total amount of N in the N_2-fixing plant. It is therefore necessary to establish as accurately as possible the resulting ^{15}N enrichment of the soil N after material of higher ^{15}N content has been incorporated. However, this cannot be established easily by chemical extraction. The main reasons are that no single compound enriched in ^{15}N can uniformly label the soil N, and that the N provided to the plant may come from different sinks with different ^{15}N enrichments. The isotope dilution principle is based on the assumption that when two sources that differ in isotopic composition are uniformly mixed, the resulting isotope composition will reflect the magnitude of the difference in the initial composition of these two sources as well as the relative amounts of each of them. Therefore, it does not apply in this classical sense.

These conditions occur only in the case of plants grown in nutrient solutions in which the source of N has been ^{15}N-labeled. For soil-grown plants, the requirements of a true isotope dilution will only be met when the soil N itself presents a natural $\delta^{15}N$ value different from that of the atmosphere. Refer to the natural abundance method presented in Chapter 4.

Use of ^{15}N-enriched materials, generally fertilizers added to the soil, results in access of fixing plants to three nitrogen sources: soil N, fertilizer N, and atmospheric N. According to the *A*-value concept of Fried and Dean (1953), a plant having several sources of nutrient will absorb this nutrient from these sources in direct proportion of the amounts available. The quantity of available nutrient can be estimated in terms of a standard amount of that nutrient added to the soil (e.g., ^{15}N-enriched fertilizer) using the relationship

$$A = \frac{B(1 - y)}{y} \tag{7}$$

where A is the amount of nutrient available in the soil, B is the amount of nutrient in the standard, and y is the proportion of the nutrient in the plant derived from the standard.

If ^{15}N-labeled fertilizers are used as the standard, y is the percentage of total nitrogen in the plant that comes from the fertilizer (%Ndff) and the A value becomes

$$A = \frac{(100 - \%\mathrm{Ndff})}{\%\mathrm{Ndff}} \times \text{rate of fertilizer N applied (kg N ha}^{-1}) \qquad (8)$$

and the proportion of total N derived from the fertilizer can be estimated as

$$\%\mathrm{Ndff} = \frac{^{15}\mathrm{N\ atom\%\ excess\ in\ plant}}{^{15}\mathrm{N\ atom\%\ excess\ in\ fertilizer}} \times 100 \qquad (9)$$

In the case of N_2-fixing plants, this also applies, but the percentage of N not derived from the fertilizer comes both from the soil N and from the atmospheric N. The problem is therefore to separate the two components.

This can only be achieved if a nonfixing plant having access to the same source of soil N as the N_2-fixing plant is used to calculate the *A* value for the soil N. The *A* value for soil + atmospheric N is then given by the fixing plant. The amount of fixed N can therefore be calculated as follows:

$$\begin{array}{c}\mathrm{N_2\ fixed}\\ (\mathrm{kg\ ha^{-1}})\end{array} = \begin{array}{c}A\text{-value}\\ \text{fixing system}\end{array} - \begin{array}{c}A\text{-value}\\ \text{nonfixing system}\end{array} \qquad (10)$$

$$\times \frac{\%\mathrm{Ndff} \times \text{total plant N (kg ha}^{-1})}{\text{fertilizer N applied (kg ha}^{-1}) \text{ fixing system}} \times 100$$

This conceptual approach, which recognizes three N sources for fixing plants, is still used in some circumstances for estimating N_2 fixation. However, its application is problematic (Vose *et al.*, 1981; Rennie and Rennie, 1983), for reasons that will be discussed later.

A modification of this *A*-value approach has been developed by Fried and Middleboe (1977). According to these authors, if identical rates of labeled fertilizer with the same ^{15}N enrichment are applied to both fixing and nonfixing systems, Eq. (10) reduces to the following:

$$\%\mathrm{Ndfa} = 1 - \frac{^{15}\mathrm{N\ atom\%\ excess\ (fixing\ plant)}}{^{15}\mathrm{N\ atom\%\ excess\ (nonfixing\ plant)}} \times 100 \qquad (11)$$

This equation is the one generally adopted for the isotope dilution method, which assumes uniform mixing of the ^{15}N fertilizer into the soil N and therefore two sources of N for the plant: soil and atmosphere. Provided that the preceding conditions for its application are met, this modification of the *A*-value method is conceptually and analytically simpler. As indicated by Eq. (11), it ignores the rate of fertilizer applied and is yield-independent. Of course, for most studies, the total N content of a crop is needed to estimate the amount of N derived from biological fixation. However, certain assumptions and requirements are necessary concerning the choice of the nonfixing control plant and application of the ^{15}N-labeled fertilizer. They will be described below.

B. Materials Required

Unlike the $^{15}N_2$ reduction method, the isotope dilution method does not require sophisticated equipment above that necessary to measure the ^{15}N content of the plant material, described in Chapters 2 and 3. However, the plant material and the design of field experiments are of great importance.

1. Source of ^{15}N-Enriched Materials

Many different forms of ^{15}N-enriched materials have been used to estimate N_2 fixation. These include ammonium salts such as sulfate, nitrate or chlorate, potassium or calcium nitrate, urea, residual ^{15}N, or organic residues labeled with ^{15}N. It has been suggested that ammonium is preferable to nitrate because much of the tracer is readily incorporated into the internal soil N cycle. On the other hand, nitrate will minimize microbial transformations. As a rule, it seems logical to add labeled N compounds in a form that is already present in the soil. It is also probable that slow-release formulations or organic matter labeled with ^{15}N will maintain a more stable enrichment of the soil. However, such materials are not always available, and most routine experiments have used ammonium sulfate without much justification.

^{15}N enrichment for legumes has ranged from as low as 3 atom% excess to as high as 97%. Because the cost of ^{15}N-labeled material increases disproportionately as the $^{15}N\%$ increases, an average of 5% enrichment is adequate for most experiments. The rate of N application and the apparatus used for measuring ^{15}N content in the plant material (emission or mass spectrometer) will govern the specific range. For symbiotic nitrogen fixation, when using a mass spectrometer a rate of 5–10 kg N ha^{-1} labeled at 5–10% excess will be sufficient. For emission spectrometry, a coefficient of 10 will have to be applied, either 30–50% ^{15}N enrichment or 50–100 kg ha^{-1}. Care should be taken not to decrease the rate of N_2 fixation; a high mineral nitrogen level in the soil is known to inhibit biological nitrogen fixation.

Methods of application of ^{15}N-labeled materials have ranged from broadcast spraying to injection of solution into soil. Because the isotope dilution method requires uniform distribution of added ^{15}N into the soil, application

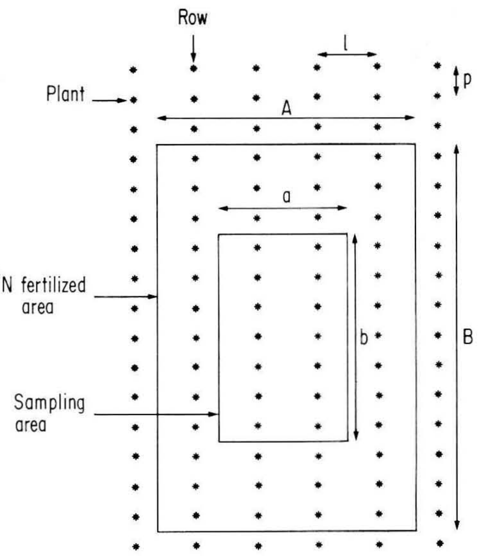

Figure 5 Subplot design for application of ^{15}N- enriched fertilizer in N_2 fixation studies.

in solution is the better choice. It has the advantage that a small amount of fertilizer can be dissolved in a large volume of water and, therefore, applied uniformly. The apparatus can be a fine garden sprayer or a laboratory plastic washbottle. The latter is preferred to avoid direct contact with the plants, which may result in burning or assimilation.

2. Plots Sizes and Design

A randomized block design with about six replicates is generally recommended. The nonfixing reference crop should be located as close as possible to the fixing crop. To reduce the cost of isotope, ^{15}N fertilizer is applied to small subplots situated in the center of a plot, the remainder of the plot being used for yield data. Figure 5 illustrates the type of design that can be adopted.

If measurements have to be made on 14 different plants of each treatment and if interplant spacing (p) is about 10 cm and interrow spacing (l) is 40 cm, the sampling area ($a \times b$) must be around 0.6 m^2:

$$a = 2 \times l = 0.8\ \text{m},$$

$$b = 7 \times p = 0.7\ \text{m}.$$

The fertilized area ($A \times B$) must be approximately 2 m^2:

$$A = a + 2l = 1.6\ \text{m}$$

$$B = b + 6p = 1.3\ \text{m}$$

It is recommended that the ^{15}N-fertilized subplots be located in a horizontal area to reduce lateral leaching.

3. Plant Material

a. N_2-fixing plants The ^{15}N isotope dilution method has been applied successfully to a great number of legume crops, both grain and forage, and even to associative N_2 fixation in grasses. The main limitation is availability of a suitable nonfixing control plant with the same growth pattern as the fixing plant. There are also some major problems with the use of long-lived or perennial plants. Unless new modifications are developed, the isotope dilution method will remain a tool for fast-growing annual plants, best suited to studies of legumes.

b. Nonfixing control plants The accuracy and precision of the isotope dilution method depends on the selection of a nonfixing reference plant. This plant should have the following characteristics:

- It should not fix nitrogen. This can be checked rapidly using the acetylene reduction assay.
- Its roots should exploit the same soil zone simultaneously with the fixing plants and, therefore, have access to the same ^{15}N label. This is very important because it has been demonstrated that the ^{15}N enrichment of the soil N does not normally remain constant but, rather, declines with time. A corollary is that the distribution of ^{15}N must be as uniform as possible within the system.
- The growing period of both nonfixing reference and fixing plants should be almost the same. They must be planted, mature, and harvested at the same time.
- Both crops should be affected in the same fashion by changes in environmental conditions such as temperature and water.
- There should be no major discrimination or uneven distribution of ^{15}N between roots and shoots of either plant.

Although ideal nonfixing reference crops do not exist, the following plants can be used for estimating N_2 fixation in legumes: a nonlegume, nonfixing plant; a nonnodulating legume; and a noninoculated legume in soil without the appropriate *Rhizobium* strains.

Studies of grain legumes such as soybean (*Glycine max*), field bean (*Phaseolus vulgaris*), or fava bean (*Vicia faba*) have used noninoculated plants in soils devoid in *Bradyrhizobium japonicum* or *Rhizobium*, nonnodulating isolines, wheat (*Triticum*), barley (*Hordeum vulgare*), and even rapeseed (*Brassica napus*). Sudan grass (*Sorghum sudanense*) is recommended by the International Atomic Energy Agency (IAEA), Vienna, Austria.

Forage legumes do not yet present suitable nonnodulating isolines; therefore, perennial ryegrass (*Lolium perenne L.*) is commonly used as a reference crop.

In the case of mixed pastures, the nonfixing companion crop in the mixture is usually used as the reference. However, it is desirable to establish that its growth patterns and especially its root characteristics match those of the legume and that no significant transfer of N from the legume to the companion crop occurs.

4. Sampling and Analysis

Equipment for sampling ^{15}N-enriched plant material in the field does not differ from that used for other field studies. Aboveground parts are clipped and separated into component parts using scissors. Belowground parts are extracted by soil coring, using manual or hydraulic augers. Roots and nodules are washed free of soil using specially designed root washing machines or thorough agitation of the roots in sieves of several mesh sizes to minimize root losses.

The material and equipment used to prepare the samples and to analyze their content in ^{15}N are the same as those described previously.

C. Description of Procedures

1. Labeling the Soil N

When fixing and nonfixing reference plants are sown according to the plot design described earlier, ^{15}N fertilizer application may proceed on the isotope subplots as follows.

A solution of labeled ammonium sulfate is prepared to provide each 2-m^2 subplot with approximately 1 g N and 0.05 g ^{15}N. This corresponds to a fertilization rate of 5 kg N ha^{-1} and a ^{15}N enrichment of 5%. The required amount of $(^{15}NH_4)_2SO_4$ is dissolved in 2 liters of distilled water and dispensed to each subplot using a spraying apparatus. This is followed by washing the area with the same amount of distilled water for even distribution of the isotopic solution. A preliminary trial on an area of identical size using the same amount of plain water may provide useful practice and also determine the soil depth reached by the water. The total amount of solution can then be modified according to the specific nature of the system under study (e.g., root growth patterns, soil characteristics).

For grain legumes, a single application is made at the time of planting, on both fixing and nonfixing reference plants. For forage legumes, due to the rapid decline in ^{15}N enrichment of the soil, equal small doses of ^{15}N fertilizer may be applied at short intervals, such as between each harvest.

Care should be taken that ^{15}N is applied in the absence of wind and when the soil temperature is not so high as to favor direct ammonia volatilization. Contact of fertilizer with plant parts should be avoided.

2. Harvesting the Plant Materials

The isotope dilution method gives an integrated value for N_2 fixation in the field. For a correct estimate, differences in ^{15}N enrichment between fixing and nonfixing plants must be significantly higher than the error in measurement. Therefore, sampling must follow ^{15}N application by a reasonably long period of time. In general, if fertilizer is applied at planting time for grain legumes or after cutting for forage legumes, plants are harvested during the pod-filling stage and at 50% flowering, respectively. To estimate intermediate rates of N_2 fixation, plants may be harvested at different periods during the growth cycle.

It has been demonstrated that different plant parts (e.g., seeds, pods, leaves, stems, crown, roots) may differ in isotopic composition. Therefore, each of them must be sampled for ^{15}N determination. To avoid time-consuming and costly analysis, it may be useful to perform preliminary analysis of different plant parts, to decide if splitting into different parts is necessary. This is generally so for grain legumes. For forage legumes, aboveground portions, flowers, and seeds are more equal in densities and more evenly represented in whole-plant sampling.

When plants have been harvested and separated, they are dried at 70°C and weighed. Subsamples are then chopped in small pieces and reduced to powder in a mill. They can then be stored until ^{15}N analysis. When possible, material with lower ^{15}N enrichment (e.g., fixing plants) should be analyzed first to avoid or minimize contamination. Equipment must be cleaned thoroughly between treatments.

Although the N content of different plant parts may be estimated from plant material harvested on the labeled subplots, yield determination may require additional sampling on the nonlabeled area, to get a representative estimate.

3. Calculations

Estimation of N_2 fixation can be made as follows. Values to be measured are as follows:

1. Dry matter yield of different plant parts (e.g., pods, stems, leaves, crowns, roots, nodules) on a per plant or per hectare basis
2. Percentage of N of each plant part
3. ^{15}N atom% of each plant part, for fixing and nonfixing plants

According to the general equation of the isotope dilution method

$$\%\mathrm{Ndfa} = 1 - \frac{{}^{15}\mathrm{N\ atom\%\ excess\ (fixing\ plant)}}{{}^{15}\mathrm{N\ atom\%\ excess\ (nonfixing\ plant)}} \times 100. \qquad (11)$$

Although a true ^{15}N atom% excess determination requires a control for both fixing and nonfixing plants consisting of plants growing without ^{15}N fertilizer, the atmospheric value of 0.3663 ^{15}N atom% is generally employed for the calculations. The difference in natural abundance is likely to be very small compared to the ^{15}N enrichment due to the fertilizer (Mariotti *et al.*, 1983). Therefore,

$$^{15}\mathrm{N\ atom\%\ excess\ in\ each\ plant\ part} = {}^{15}\mathrm{N\ atom\%} - 0.3663.$$

A weighted ^{15}N atom% excess for the whole plant (fixing and nonfixing) is derived from the equation

$$\mathrm{WAE} = \frac{\mathrm{AE}_{(a)} \times \mathrm{TN}_{(a)} + \mathrm{AE}_{(b)} \times \mathrm{TN}_{(b)} + \ldots \mathrm{AE}_{(n)} \times \mathrm{TN}_{(n)}}{\mathrm{TN}_{(a+b+\ldots n)}} \qquad (12)$$

where WAE is weighted ^{15}N atom% excess, AE is ^{15}N atom% excess in different plant parts represented as a, b, ..., *n*, and TN is total N in parts a, b, ..., *n*.

Knowing the %N derived from fixation, total N_2 fixation is calculated as

$$\mathrm{N_2\ fixed\ (kg\ ha^{-1})} = \frac{\%\mathrm{Ndfa} \times \mathrm{total\ N\ in\ fixing\ crop\ (kg\ ha^{-1})}}{100}. \qquad (13)$$

D. Comments

The major limitation of the isotope dilution method lies in the main assumption that the ^{15}N fertilizer is uniformly mixed with soil N. In fact, the soil ^{15}N enrichment changes with time and space, and the use of the method is totally dependent on the comparison between fixing and nonfixing reference plants. Choice of a proper nonfixing system is therefore of prime importance. It must sample N with identical isotopic composition in the soil and fertilizer pools as does the fixing system. As mentioned for grain legumes, several nonfixing systems exist. With forage legumes, nonnodulating isolines are not always available. When using nonlegumes such as grass for reference, one cannot be certain that root growth pattern and therefore N uptake will match between tested and reference crop. Another requirement of the method is that the amount of N added has to be the same in both systems. N addition must also be small, because high soil mineral N levels normally inhibit N_2 fixation. In nitrogen-poor soils, the nonfixing system may therefore not get enough N to ensure adequate growth. This limits the use of the isotope dilution method. In such cases, an alternative is to use the *A*-value method. This variation of the isotope dilution method has the advantage that fixing and nonfixing systems may be given different rates of fertilizers. However, it has the disadvantage that it is yield-dependent, which infers a low degree of precision, and so is not very reliable at low rates of fertilizers (Rennie and Rennie, 1983) as required for N_2 fixation studies. This method further assumes that the availability ("*A*-value") of unlabeled soil N does not change with increasing rates of added fertilizer; this has not always been found to be true. In common with the isotope dilution method, the *A*-value method also suffers from having to match the soil N uptake patterns of the test and control crops. For these reasons, the "*A*-value" concept, which was important in development of the methodology leading to the isotope dilution method, is less reliable and far more complex. In most cases, it has been superceded by the latter in N_2 fixation studies.

The inherent simplicity of isotope dilution makes it the method of choice for general purposes. It has been found to be more precise than any other method for estimating N_2 fixation. The greater the proportion of nitrogen in the test crop that is derived from atmosphere, the greater is the difference between ^{15}N enrichment of the fixing and nonfixing plant, and the smaller is the error in the estimates. Its application to legumes is most obvious. However, successful studies have been performed with nonfixing plants having an associative N_2 fixation, such as grass, providing a suitable nonfixing reference crop can be found. This suitable reference may be available for annual crops such as wheat, which have nonfixing genotypes of similar physiological growth pattern (Rennie *et al.*, 1983). The method has proven adequate for selecting genotypes that respond differently to inoculation by N_2-fixing microorganisms. However, there are major problems with long-growing or perennial grass. These have been discussed in a recent review (Boddey, 1987).

Another application of the isotope dilution method is to provide information on the partitioning of fixed N among different plant parts. Combining ^{15}N isotope dilution with a comparison of nodulating and nonnodulating plants can allow one to demonstrate that fixed N has a fate different from that of N derived from soil.

In conclusion, the main disadvantage of the isotope dilution method is its uncertainty, but despite this, it is the only easy and reliable method to obtain an integrated estimate of N_2 fixation in the field, providing certain requirements are met.

IV. GENERAL COMMENTS

The two methods described in this chapter, which are based on artificial ^{15}N enrichment of atmospheric N ($^{15}N_2$ reduction) or soil N (isotope dilution), are the most commonly used in N_2 fixation studies; however, they do not have the same objectives. The former is restricted to short-term laboratory or greenhouse measurements, and the latter to long-term integration of N_2 fixation in the field. Both methods can be adopted for complementary work in N_2 fixation. The use of ^{15}N isotope is not restricted to researchers having ^{15}N determination facilities, because many commercial laboratories now analyze samples from both methods at reasonable cost.

As in most isotopic studies, ^{15}N techniques applied to N_2 fixation are destructive and, as such, they require a fair amount of material to give meaningful data. This is especially true when periodical sampling is required. The $^{15}N_2$ reduction method is somewhat more artificial because it uses a closed incubation system. However, being a direct measurement, it does not generally require a control. The isotope dilution method is far less complicated but relies on differences between fixing and nonfixing control plants. Also, the disturbance of the soil N by ^{15}N fertilizer addition is difficult to control.

A common concern that arises in the use of ^{15}N-enriched material is that it may involve contamination, even if all precautions are taken for cleaning the equipment used for sample preparation and analysis. It must be emphasized that when laboratory space or equipment has been used for ^{15}N-enriched material it is no longer suitable for natural-abundance studies. Most material has a ^{15}N "memory," and its usage may lead to erroneous data. However, methods based on natural abundance are not yet capable of replacing ^{15}N-enrichment methods for serving the same objectives. Therefore, a choice must be made before introducing a ^{15}N isotope method in a laboratory.

A special variation of the isotope dilution method is to use ^{15}N-depleted material to label the soil N, thus lowering the ^{15}N ratio of N derived from the atmosphere in the fixing plant. Based on the same principles as isotope dilution in the presence of ^{15}N-enriched material, it presents the same disadvantages (e.g., the need to find a nonfixing crop that matches the soil N uptake of the fixing crop). Besides, the great dilution of the tracer, which has a maximum difference from other sources of 0.36 atom%, requires a very great precision in ^{15}N determination. It is therefore unlikely that methods based on ^{15}N-depleted materials will be useful in N_2 fixation studies. The only advantage is that such methods are less contaminating than methods using ^{15}N-enriched materials.

In conclusion, the potential of ^{15}N-aided methods and the concepts of their use have not yet been fully investigated. Improvement of the techniques of ^{15}N determination together with the decline in costs, will contribute to widespread adoption of these methods in the fascinating and crucial field of N_2 fixation.

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6

Nitrogen Fixation in Flooded Rice Soils and Aquatic and Sediment Systems

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I. INTRODUCTION

A. System Description

1. Flooded Rice Soils

Flooded rice fields give favorable conditions for biological nitrogen fixation owing to the presence of (1) the surface floodwater, which harbors photodependent nitrogen-fixing microorganisms, (2) anaerobic or microaerobic soils, and (3) microaerobic rice rhizosphere. The highest activity is exhibited by the floating aquatic fern *Azolla,* associated with cyanobacteria (blue-green algae), and the lowest by anaerobic soil, without organic amendment. Due to the nitrogen-fixing activities in flooded rice soils, a higher N fertility of such soils has been maintained compared to that of dryland soils (Watanabe and Roger, 1984).

Despite the well-recognized role of flooding and anaerobiosis in nitrogen fixation in flooded rice soils, knowledge on the quantity of nitrogen fixation in this system is still limited due to difficulties in methodology (Watanabe and Roger, 1984). Measurement of nitrogen-fixing activities primarily aims at knowing the contribution of biological nitrogen fixation to the N balance of the soil–plant system and to the N nutrition of rice *in situ*. Although the ^{15}N method is more expensive than acetylene reduction assay (ARA), it gives a more quantitative measurement. The fate of biologically fixed N in N cycling is traced only by using ^{15}N.

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2. Lakes and Oceanic Systems

In oligotrophic systems, N is a major limiting nutrient for primary production. Bacteria (including cyanobacteria) are the major diazotrophic groups. They are planktonic, particulate-bound, associated with aquatic macrophytes, and benthic. Cyanobacteria operate active nitrogen fixation in sites where nitrogen limits primary production and sufficient light is available. The highest activities are exhibited by *Trichodesmium* sp. in the pelagic ocean and *Calothrix crustacea* in coral reefs and in algal mats in tropical seagrass meadows. A *Spartina*–bacteria association in saltmarshes is the most active site for heterotrophic bacterial nitrogen fixation in saline habitats.

B. Choice of Incubation Period and ^{15}N Analyzers

The methods chosen for quantifying nitrogen-fixing rates depend on the activity. The acetylene reduction method has been widely used due to its simplicity and shorter exposure time, although its problem as a quantitative tool remains. Because of the lower sensitivity of the ^{15}N-labeled N_2 gas method, a longer incubation period is required. The minimum period of exposure to various systems is determined by the detection limit of ^{15}N in the samples, the nitrogen-fixing activity, and the ^{15}N abundance in the N_2 gas used. The detection limits, expressed as a dilution of ^{15}N in the samples fed with 99 atom% ^{15}N containing N_2 gas, are 1.5×10^{-6} (140 μg N sample) and 3.3×10^{-6} (35 μg N sample) by ratio mass spectrometry. Those by single-scanning mass spectrometry are 1.0×10^{-5} (140 μg N sample) and 1.0×10^{-4} (35 μg N sample). The detection limit by emission spectrometry is 3.0×10^{-4} in samples with 10 μg N. For finding the incubation period or suitable methods of detecting ^{15}N enrichment, nitrogen-fixing activity is expressed as N fixed per hour per N in the sample (Tables I and II). To calculate the exposure period, the most sensitive ratio mass spectrometer and the least sensitive emission spectrometer values are taken (Tables I and II). The exposure time in Table I is calculated when the labeled N_2 gas has 99–100 atom% ^{15}N. When ARA ranges from 0.4 to 10 nmol C_2H_4 hr^{-1}/sample, which contains 140 μg N, only the ratio mass spectrometer can be used for detecting the incorporation of ^{15}N within an hour.

It is preferable to use an exposure period as short as possible, because some nitrogen-fixing systems are fragile and longer incubation may create artefacts and may not represent *in situ* activity. For longer incubation, special care is needed to avoid side effects such as the increase of temperature inside the enclosed assay chamber by solar radiation and the change of isotope ratio and gas composition in the head space (the depletion of

Table I
Nitrogen-Fixing Rate of Various Nitrogen-Fixing Agents in Flooded Rice Soils and Minimum Period of Exposure to $^{15}N_2$ Gas

System	Agent	N_2-fixing rate	Total N	Activity (hr^{-1})	Exposure time[a] Required ^{15}N dilution up to 4×10^{-3} (A)	3.3×10^{-5} (B)
Floodwater and surface soil	Cyanobacteria during a bloom	<7 mg N m^{-2} hr^{-1} (1 mmol ARA m^{-2} hr^{-1})	3 g N m^{-2}	2.3×10^{-3}	2 hr	>1 min
	Azolla–Anabaena	120–600 μ N (g dry weight)$^{-1}$ hr^{-1} 1–5 μmol ARA (g fresh weight)$^{-1}$ hr^{-1}	40 mg N (g dry weight)$^{-1}$	$3.8–15 \times 10^{-3}$	63–16 min	>1 min
Anaerobic soil without amendment	Bacteria	0.1 μg N (g soil)$^{-1}$ day^{-1}	1.5 mg N (g dry soil)$_{]1}$	$<2.8 \times 10^{-6}$	>60 days	1 day
Anaerobic soil with amendment	Bacteria (1% glucose)	4.25 μg N (g soil)$^{-1}$ day^{-1}	1.5 mg N (g dry soil)$^{-1}$	1.2×10^{-4}	33 hr	16 min
Associated with rice	Bacteria	85 μg N (400 g soil)$^{-1}$ day^{-1}	1.8 mg N (g dry soil)$^{-1}$	5.0×10^{-6}	33 days	7 hr
		6.2 μg N (2.9 g root)$^{-1}$ day^{-1}	4.5 mg N (g dry root)$^{-1}$	2.0×10^{-5}	8 days	1.5 hr
		140–220 μg N (root + leaf sheath)$^{-1}$ day^{-1}	5.5 g root + leaf sheath (34 mg N)	$1.7–2.7 \times 10^{-4}$	14–24 hr	7–10 min
Associated with deepwater rice	Cyanobacteria + bacteria	250 μg N (leaf sheath of floating parts)$^{-1}$ day^{-1}	36 mg	2.9×10^{-4}	13 hr	7 min
		880 μg N (plant)$^{-1}$ day^{-1}	540 mg N	6.8×10^{-5}	2.5 days	30 min

[a] Ten times the detection limit described in the text by (A) emission spectrometer (sample size 10 μg N) and by (B) ratio mass spectrometer (sample size 140 μg N); 99 atom% $^{15}N_2$ is used.

Table II
Nitrogen-Fixing Rate of Various Nitrogen-Fixing Agents in Aquatic Ecosystems and the Minimum Period of Exposure to $^{15}N_2$ Gas

System	Agent	N_2-fixing rate	Activity (hr^{-1})	Exposure time[a] Required ^{15}N dilution up to 4×10^{-3} (A)	3.3×10^{-5} (B)
Seawater	*Trichodesmium*	5–100 μg N m^{-2} hr^{-1}	$<12\ (2.2) \times 10^{-3}$	1.8 hr	<1 min
	Richelia intracellularis	—	$<0.64\ (0.33) \times 10^{-4}$	12 hr	<6 min
Coastal water	*Anabaena torulosa* and others[b]	1–200 mg N m^{-2} day^{-1}	$0.1\text{–}2.4\ (1.3) \times 10^{-3}$	3.1 hr	1.5 min
Coral reef and seagrass meadow	*Calothrix* sp.	<200 mg N m^{-2} day^{-1}	$<10\ (5.0) \times 10^{-3}$	48 min	1 min
Coastal sediment		1–890 ng N (g soil)$^{-1}$ day^{-1} 6–400 μ N m^{-2} day^{-1}	$<1.9\ (1.1) \times 10^{-5}$	15 days	3.0 hr
Saltmarsh sediments[c]		<1000 ng N (g soil)$^{-1}$ day^{-1} <300 mg N m^{-2} day^{-1}	$<2\ (1.1) \times 10^{-5}$	15 days	3.0 hr
Lake cyanobacteria	*Anabaena* sp. *Aphanizomenon flos-aquae* *Calothrix* sp. *Gloeotrichia echinulata* *Nostoc* sp.	5–100 μg N m^{-2} hr^{-1}	$<5.6\ (2.8) \times 10^{-3}$	1.4 hr	1 min
Lake sediment		10–100 ng N (g soil)$^{-1}$ day^{-1}	$<2.1\ (1.1) \times 10^{-5}$	15 days	3 hr

[a] Ten times the detection limit described in the text by (A) emission spectrometer (sample size 10 μg N) and by (B) ratio mass spectrometer (sample size 140 μg N); 99 atom% $^{15}N^2$ is used. The average values are given in parentheses.

[b] Includes *Anabaena variabilis, Calothrix aeruginea, Microchaeta* sp., and *Rivularia* sp.

[c] Sedimentary organic nitrogen is assumed to be 0.2% (dry weight basis) for calculation of activity.

carbon dioxide and the increase of oxygen in the photosynthetic system, and oxygen elimination and accumulation of carbon dioxide and methane in the heterotrophic system). Shorter incubations are, of course, less useful if an objective is to quantify nitrogen fixation over longer periods. In such a case, repeated assays are required.

The ^{15}N dilution technique is used to estimate the contribution of biologically fixed N in the plant. This method can measure the contribution of nitrogen fixation to N nutrition of plants such as *Azolla* and rice over time periods longer than those appropriate for the measurement of $^{15}N_2$ incorporation.

Because fluctuations of activities in time and space are quite great in aquatic systems (particularly in flooded soils where artificial disturbance is caused by farming operations), proper sampling strategies, as described in Section III.A, should be developed before applying ^{15}N methods.

II. N_2 GAS PREPARATION AND ^{15}N ANALYSIS

N_2 gas purchased or prepared from labeled ammonium salt contains impurities such as nitrogen oxides and ammonia. Cleaning of the gas before using the $^{15}N_2$ is therefore absolutely essential. The determination of the atom% excess of ^{15}N in the gas phase in the assay chamber is also essential.

A. Preparation and Purification of $^{15}N_2$ Gas and Transfer to the Assay Bottle

Methods for the preparation of $^{15}N_2$ by oxidation of ammonium ^{15}N by alkaline hypobromite, and for the purification of such gas or of commercially purchased $^{15}N_2$, have been reported by Ohyama and Kumazawa (1981). They are described in detail in Chapter 5, Section II.B.2.

A modification of the method, which has been developed for use on-board ship, is as follows.

1. Materials Required

1. Gas exchange manifold, as shown in Fig. 1.
2. KOBr solution: Mix 100 g Br_2 with 741 g KOH-KI solution (H_2O : KOH : KI = 600 : 140 : 80 w/w). The bromine should be added slowly at <5°C and any precipitate filtered off later.
3. Reagent grade ^{15}N-labeled ammonium sulfate.

2. Procedure

1. The vessel system (Fig. 1j) is evacuated to a pressure $<10^{-3}$ mm Hg (1.33 Pa) by using a rotary pump. After closing the stopcocks, ^{15}N-labeled

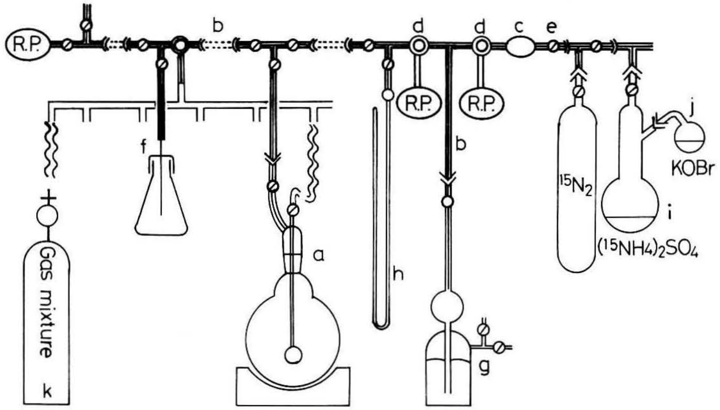

Figure 1 Gas exchange apparatus used for shipboard experiments. (a) Glass bottle for incubation of water sample; (b) capillary tube (1 mm ID); (c) H_2SO_4 trap; (d) three-way stopcock; (e) stopcock; (f) hypodermic needle and serum bottles; (g) mercury Toepler pump; (h) mercury manometer; (i) ^{15}N-$(NH_4)_2SO_4$ solution; (j) KOBr; (k) gas cylinder. R.P., rotary pump.

ammonium sulfate powder is mixed with KOBr by reversing the vessel (Fig.. 1j) to convert NH_3 to N_2.

2. The $^{15}N_2$ gas produced should be purified from $^{15}NH_3$ by passing it through an acidified glass wool trap (Fig. 1c). The trap is packed with a glass wool acidified with 0.5 *N* H_2SO_4. $^{15}N_2$ gas still contains a trace of NO_x. It is not always necessary to reduce NO_x to N_2 by passing through a Cu furnace at 400°C, because the effect of NO_x is undetectable.

3. $^{15}N_2$ gas is introduced into the incubation bottles via a capillary tube of 1 mm ID by using a mercury Toepler pump (Fig. 1g). The pressure of $^{15}N_2$ gas is measured with the mercury manometer. Residual $^{15}N_2$ gas is recovered into the $^{15}N_2$ container by using the same Toepler pump.

B. Preparation of Atmospheric Samples for Emission Spectrometer Analysis

1. Materials Required

1. Kumazawa's gas preparation unit for emission spectrometer (Shoukou Tushou Company Ltd., Tokyo) (Yoneyama and Kumazawa, 1974).
2. Glass discharge tube, Pyrex glass, 4 mm diameter.
3. Liquid nitrogen.
4. Reduced Cu wire pieces, freshly prepared by heating at 300°C under hydrogen.
5. Electric furnace.

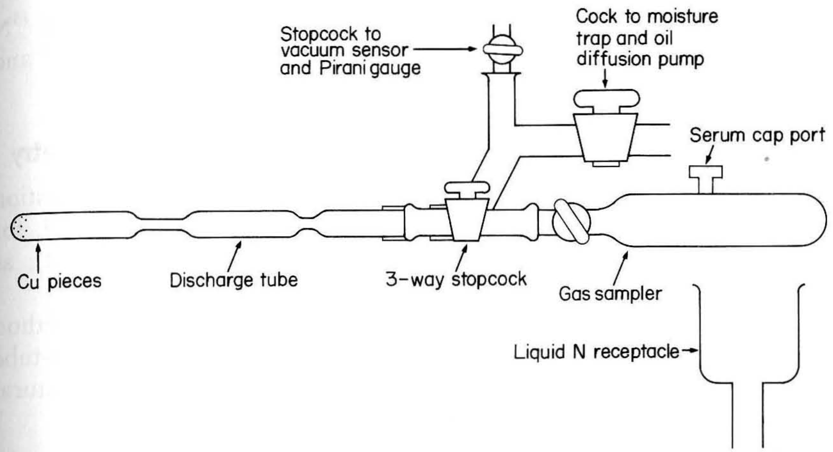
Stopcock to vacuum sensor and Pirani gauge
Cock to moisture trap and oil diffusion pump
Serum cap port
Cu pieces
Discharge tube
3-way stopcock
Gas sampler
Liquid N receptacle

Figure 2 Apparatus for making a discharge tube to measure ^{15}N content in N_2 gas by emission spectrometry.

6. Gas sampler, as shown in Fig. 2.
7. Emission spectrometer (such as ^{15}N analyzer N 150, Japan Spectroscopic Company Ltd., Hachiouji, Tokyo).

2. Procedure

1. Connect the gas sampler to Kumazawa's gas sample preparation unit for emission spectrometry as shown in Fig. 2. Connect the discharge tube containing a few pieces of reduced Cu inside. Heat the discharge tube with a hand torch. Freeze the gas sampler by liquid nitrogen, evacuate the unit, and then raise the gas sampler portion from the liquid nitrogen receptacle and thaw it with a hair dryer. Repeat freezing, evacuation, and thawing several times. Evacuate the unit until 0.003 torr (0.4 Pa) and check for leakage.
2. Take a gas sample of the atmosphere to be analyzed by using a gas-tight syringe. Close the stopcock of the gas sampler, and then transfer the sample gas to the gas sampler through its serum cap port.
3. Freeze the gas sampler with liquid nitrogen, and then close the cock leading to the water trap and diffusion pump; turn the three-way cock to close the way to the discharge tube. While freezing the gas sampler, slowly open the stopcock of the gas sampler until the pressure read by the Pirani meter increases to 2–5 torr (270–670 Pa). While maintaining this pressure, open the three-way cock to the discharge tube.
4. Using a torch, cut the discharge tube off the unit. Heat it in a furnace at 500°C for 1 hr.

5. Determine the $^{15}N/^{14}N$ ratio by emission spectrometry. When the ^{15}N isotope ratio is high, use the ratio of peak heights between mass 29 and mass 30.

C. Preparation of Sample N_2 Gas for Ratio Mass Spectrometry

Organic nitrogen in a sample is converted to N_2 gas through wet digestion (Kjeldahl digestion and KOBr oxidation; see Chapter 2, Section III, for details) or the dry combustion method (modified Dumas method), as follows.

Vacuum-dried sample materials are subjected to a combustion method that has less contamination than the digestion method. A double-tube method developed by Minagawa *et al.* (1984) is commonly used for natural ^{15}N abundance studies.

1. Materials Required

1. Quartz tube (9 mm diameter and 4 or 6 mm length) (Fig. 3)
2. CuO wire, Cu wire, Ag foil, and Pt wire
3. Cajon metal fittings (Fig. 3): Ultra Torr Union (SS-6-UT-6 and SS-6-UT-8) and flexible tube (321-8-X-1)
4. Muffle furnace
5. Vacuum manifold for separation of N_2 gas (Fig. 4)

2. Procedure

1. The combustion apparatus consists of a double tube, which is preheated at 850°C for 2 hr to eliminate organic contaminants (Fig. 3). Organic materials are placed in the inner tube (Fig. 3). One gram of CuO wire pieces (purified for 2 hr at 900°C) is placed into the tube followed by a piece of Ag foil (0.05 mm thick, 4 × 50 mm, pretreated at 850°C) together with Pt wire, if necessary.
2. The tube is then put into the outer quartz tube with 1 g of Cu wire pretreated with H_2 at high temperature (<500°C).
3. The whole tube is attached to the vacuum manifold via a Cajon fitting and evacuated several hours to remove air and water in a sample and then sealed at 18 cm length.
4. The sample tubes are then combusted in a muffle furnace to 500°C for ½ hr and then elevated to 850°C for 2 hr. The sample tube in the furnace is allowed to cool for 18 hr (see comment 2 in Section II.C.1.3).
5. After combustion, the tube is attached to a vacuum manifold or an inlet system of a mass spectrometer (Fig. 4) and cracked to introduce the N_2 gas using a metal fitting as illustrated in Fig. 4. In this case, the quartz tube passes through the metal bellow so that it can be cracked there. CO_2 and H_2O in the sample tube are condensed by dipping the bottom of the tubes into liquid nitrogen.

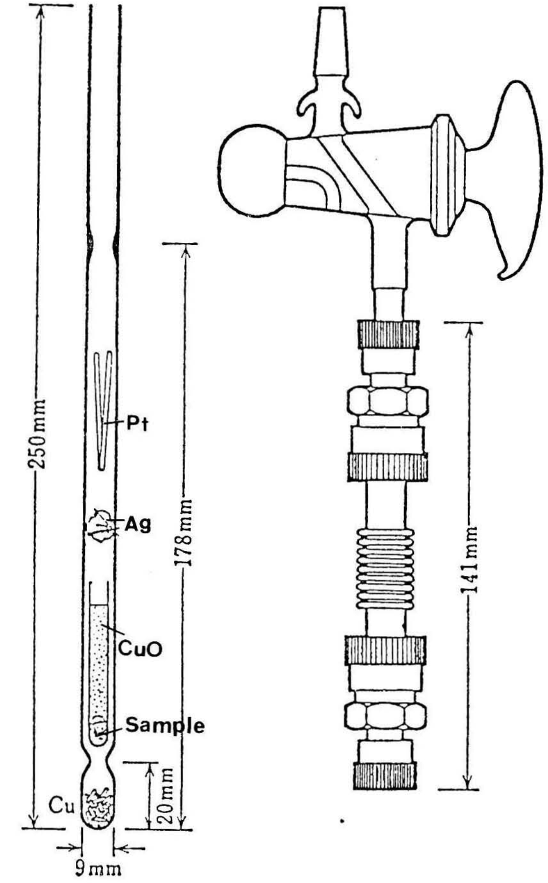

Figure 3 Combustion apparatus made of quartz tube.

6. N_2 gas is concentrated in a vacuum system by using a mercury Toepler pump or a molecular sieve (MS) trap. The amount of N_2 gas is determined volumetrically using a mercury manometer.
7. N_2 gas is sealed in a 6-mm glass tube for ^{15}N analysis.

3. Comments on Procedure

1. A sample larger than 20 mg dry organic matter destroys a quartz tube during combustion.
2. Quick elevation of combustion temperature sometimes destroys a quartz

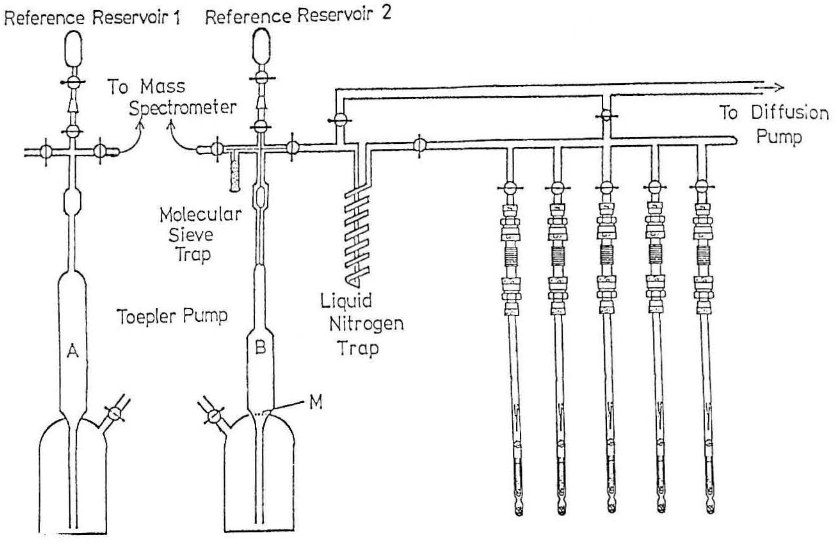

Figure 4 Gas inlet system of a mass spectrometer for ratio mass spectrometry.

tube. Cooling in a muffle furnace is important to convert CO to CO_2 and NO_x to N_2 at lower temperatures.

3. The amount of N_2 gas can be quantified in the inlet system of a mass spectrometer. Sample N_2 gas is collected in a MS (MS 5A, 5 tips) cold finger dipped in liquid nitrogen, and then the MS trap is heated up to 150°C for 3 min to desorb sample gas quantitatively. It is then expanded to a constant volume of the inlet system by setting the mercury level of the Toepler pump B at mark M (Fig. 4). The amount of N_2 gas can be determined by measuring a peak height of mass-to-charge ratio 28 with ±4 μg N SD at a 14 μg N level (Wada *et al.*, 1977).

4. In this method, the combustion yield of N_2 gas from organic N is almost 100% with negligible blank (<3 ± 2 μg N). The reproducibility is good enough to permit application of the present technique to $^{15}N_2$ tracer experiments in which small amounts of particulate samples (140 μg N) are generally used.

4. Calculation

Rate constant (V, hr^{-1}) is calculated by the following equation:

$$V = \frac{p}{N_t} = \frac{\Delta A_N/\Delta t}{\Delta A_{N_2}}$$

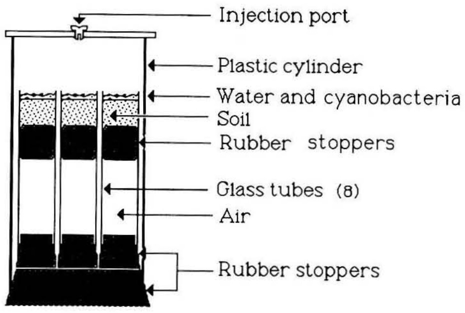

Figure 5 Incubation chamber for cyanobacteria.

where p is nitrogen fixed (μg N/h/sample), N_t is total N of a sample (μg N), ΔA_N is atom% excess ^{15}N of a sample, ΔA_{N_2} is atom% excess ^{15}N of tracer $^{15}N_2$ in a gas phase, Δt is time of exposure (hr).

5. Comment on Calculation

In ratiometry, ^{15}N content is obtained by the per mil deviation from a standard, as defined by the following equation:

$$\partial^{15}N‰ = [(R_s/R_{air}) - 1] \times 1000,$$

where R_s is $^{15}N/^{14}N$ and atmospheric nitrogen is used as a standard (0.0‰). Atom% of ^{15}N in atmospheric nitrogen is 0.3650% and its $R = 0.003663$.

$$A_S\% = \frac{^{15}N}{^{14}N + {}^{15}N} \times 100 = \frac{100R_{air}(\partial^{15}N_S/1000 + 1)}{1 + R_{air}(\partial^{15}N_S/1000 + 1)},$$

where A_s denotes atom% of ^{15}N of a sample. By this equation, A_s can be calculated from the $\partial^{15}N$ value of a sample.

III. FLOODED RICE SOILS

A. $^{15}N_2$ Incorporation in Floodwater–Surface Soil of Flooded Rice Soils

1. Materials Required

1. 2.0-cm-diameter, 15-cm-high glass tubes, 50–100 pieces.
2. 8.0-cm-diameter, 20-cm-high transparent acrylic tubes, with a rubber stopper at the bottom and an injection port at the top (see Fig. 5). One-liter Hamilton injection syringes may be useful.

3. Fluorescent light incubator box with five 20-W lamps on the top and eight 15-W lamps at each side.

2. Procedure

1. Push down a 2.0-cm-diameter sampling tube to the soil surface with gentle alternate twisting until the bottom of the tube reaches a depth of 3 cm below the soil surface. A marking on the tube facilitates this. To avoid muddiness in the water, which reduces light penetration in the samples, all tubes should be inserted first. Insertion of tubes should be made from the dike or from wooden boards placed across a field plot. After inserting the necessary number of tubes (50–100), they should then be removed with minimum disturbance of the soil (see comment 1 in Section III.A.3). Place a stopper on the top of each tube. Rotate each tube to enlarge the hole in the soil outside the tube. Lift the tube. If the soil is too soft to be retained inside the tube, support the tube at the bottom with fingers before lifting it from the soil. Then place a rubber stopper at the bottom of the tube. This stopper should have a maximum diameter almost similar to the inside diameter of the tube to facilitate pushing the bottom stopper up into the tube.
2. After bringing the tubes to the laboratory, allow the samples to stand for a few hours to let planktonic algae settle on the soil surface. Then, remove floodwater until it is a few millimeters deep on the soil surface. Push up the bottom stopper inside the tube until the upper surface of the floodwater reaches the top of the tube. Place another rubber stopper in the bottom of the tube to reduce the dead volume inside the incubation chamber. Place seven core tubes in the incubation chamber as shown in Fig. 5. Then, seal the chamber. A 1-liter Hamilton syringe can be used as a chamber.
3. Evacuate the incubation chamber to 38 kPa (290 mm Hg), let it remain for 2 min, and introduce $^{15}N_2$ gas from the gas reservoir until a slightly positive pressure is attained. Open the injection port once to equilibrate with the ambient air atmosphere. Take a gas sample to determine initial ^{15}N abundance in the gas phase just before incubation (see comment 2 in Section III.A.3).
4. Place samples in the fluorescent light incubator box in the laboratory or in a temperature-controlled room. Light illumination should be about 20,000 lx (84 W m^{-2}), temperature should be 30°C, and the incubation period should be about 1 hr (see comment 3 in Section III.A.3).
5. Just before the end of the incubation, take a gas sample for the determination of ^{15}N abundance in the gas phase (Section II.B). Soil and floodwater are digested by Kjeldahl digestion after converting nitrate to ammonium.
6. Express the results per unit area.

3. Comments

1. Soil microbial populations and their activities show a generally aggregated distribution. The activities or populations within a small area are more highly correlated with each other than with those at a distant site. To avoid this effect, the small surface area is more suitable for core sampling than a big area. With a given total sample area, variation is less when a larger number of sampling cores of smaller size are taken than when a smaller number of sampling cores of larger size are used. Thus, seven cores are pooled in a cylinder instead of taking a soil core by a cylinder directly.

In cases where cyanobacteria were visible to the naked eye, the pooled seven core samples had a coefficient of variation of 30% by ARA (P. A. Roger, ORSTOM, personal communication). The sampling strategy should be preliminarily determined by ARA assays.

2. The partial pressure of N_2 gas should be 80 kPa. Use of He or Ar with N_2 to decrease the use of labeled gas is not recommended. A N_2 gas partial pressure lower than that of ambient air may decrease the activities because gas diffusion into water-saturated soil limits the activity.
3. Incubation under the sun is not recommended because the temperature inside the incubation tubes increases to 50°C within 2 hr and, hence, inhibits the activities. In ARA assays, this glasshouse effect can be eliminated by shortening the exposure period. The longer incubation required for $^{15}N_2$ gas incorporation prohibits incubation under the sun.

B. $^{15}N_2$ Incorporation Associated with Wetland Rice

Incubation can be made with or without enclosure of the rice plant foliage in the chamber.

1. Incubation of the Whole Rice Plant within the Chamber

a. *Materials required*

1. Rice grown in flooded pot.
2. Incubation chamber, as shown in Fig. 6.
3. 50 m*M* K_2CO_3 and 100 m*M* $KHCO_3$ solution.
4. Apparatus to analyze CO_2 concentration (gas chromatograph with thermal conductivity detector or portable infrared CO_2 analyzer).

b. Procedure *Rice growth:* Rice is grown in pots, preferably in specially designed pots that can be assembled in the incubation chamber. When only nitrogen fixation associated with rice is determined, the surface of the pot should be covered by black cloth or aluminum foil to prevent algal growth.

The field-grown rice can be brought to the pot about a week before assay (Ito *et al.*, 1980).

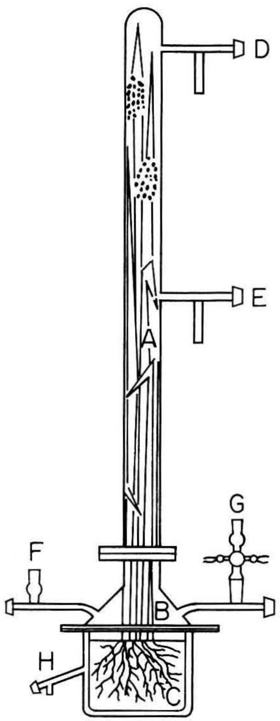

Figure 6 Incubation chamber for wetland rice. (A) Shoot compartment; (B) middle compartment; (C) root compartment; (D) circulating gas inlet; (E) circulating gas outlet; (F) $^{15}N_2$ gas inlet; (G) gas sampler and three-way cock to vacuum pump and vacuum manometor; (H) drain. [Reproduced, with permission, from Yoshida, T., and Yoneyama, T. (1980). Atmospheric dinitrogen fixation in the flooded rice rhizosphere as detected by the N-15 isotope technique. *Soil Sci. Plant Nutr.* **26**, 551–559.]

Gas exchange: The rice plant is enclosed in the chamber. The gas-tight incubator containing the rice plant is evacuated with a vacuum pump. When gas bubbles begin to emerge from the flooded soil, the chamber is maintained under this condition for several minutes. The labeled gas is stored in a large (500–1000 ml) syringe and introduced to the inlet (Fig. 6F) through a three-way stopcock, one arm of which is connected to a vacuum meter and vacuum pump (Fig. 6). The gas sample is taken from the inlet (Fig. 6G).

CO_2 concentration: The gas coming out of the outlet (Fig. 6E) is circulated by a pump and is passed through the K_2CO_3 and $KHCO_3$ solution. The ratio of K_2CO_3 to $KHCO_3$ is adjusted to give 800 ppm CO_2 concentration by monitoring its concentration from time to time.

Incubation: It is preferable to incubate the chamber in a temperature-controlled room. The period of incubation may vary from 6 to 13 days. To check if enclosure of the rice plant may damage its growth, other pots with rice should be kept at the same place without the enclosure.

Termination: At the end of the experiment, the gas samples are taken to determine ^{15}N abundance in the N_2 of the gas phase (Section II.B). The composition of other gases (O_2, CO_2, and total N_2) should be determined. As reported by Yoshida *et al.* (1983) and Eskew *et al.* (1981), rice can be grown further to see the transport of fixed N to the aboveground portion.

c. *Comments* Because N_2 gas is transported to the root zone through the aboveground portion, as shown by Yoshida and Yoneyama (1980), the whole plant should be enclosed. The enclosure immediately reduces CO_2 concentration in the chamber. Eskew *et al.* (1981) used a sophisticated method to provide CO_2 constantly by an automatic feedback supply. A portion of the gas flow is passed through an infrared gas analyzer, and a solenoid valve to feed CO_2 is opened depending on the concentration of CO_2. During the night, the gas flow is switched to a route including soda lime. Ito *et al.* (1980) used interval feeding of CO_2 during the day. Another problem is oversaturation of moisture inside the chamber. The plant leaves attached to the inside wall of the chamber often show necrosis. Eskew *et al.* (1981) and Ito *et al.* (1980) chilled the outlet of the gas to remove excess moisture and cool the inside. Systems for the automatic control of temperature, CO_2, and water vapor are described by Warembourg and Kummerow (1991) of this series. Another problem is the increase in pressure due to evolution of O_2. By venting to the atmospheric pressure, pressure control is easily done. The method of Yoshida and Yoneyama (1980) is preferable because of its simplicity.

2. Incubation of Cut Rice Hill in the Chamber

Because the enclosure of the entire rice plant poses many technical difficulties, a simple method is to enclose only the cut hill (Gowda and Watanabe, 1985).

a. *Materials required*

1. Rice plant grown in the field or in pots.
2. Glass jar and apparatus, as described in Fig. 7.

b. *Procedure*

1. Cut the rice plant at 5 cm from the soil surface. Algae and weed on the soil near the rice hill and at the base of the rice hill are removed by hand.

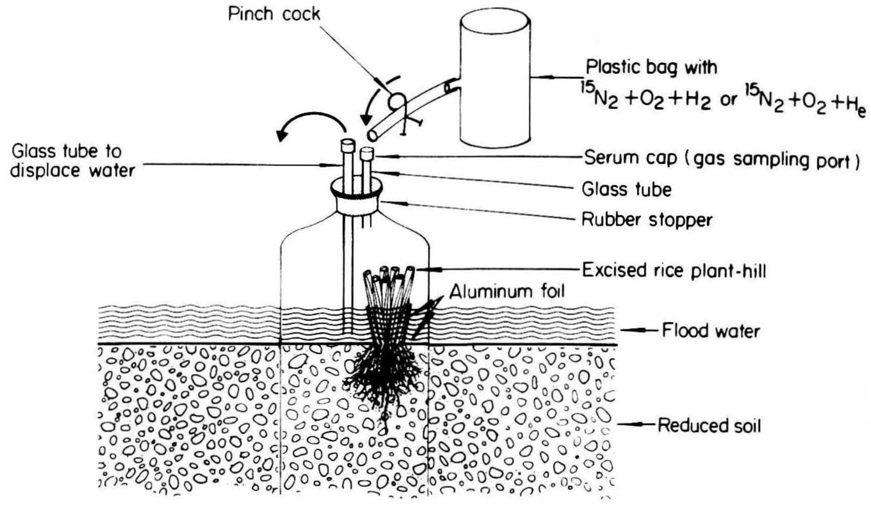

Figure 7 Incubation of rice plant hill with $^{15}N_2$ gas. [Reproduced, with permission, from Gowda, T. K. S., and Watanabe, I. (1985). Stimulating effect of hydrogen on N_2 fixation in association with field-grown wetland rice. *Can. J. Microbiol.* **31**, 503–507].

2. Insert the jar so that its lower rim extends 15 cm into the soil. The air space above the floodwater is filled by demineralized water.
3. Prepare gas consisting of 70–80% $^{15}N_2$ and 20% O_2 and He as balancing gas in a plastic bag and introduce it into the jar through the inlet. Water is displaced and expelled through the outlet tube. The volume of gas is such as to expose the cut end of the rice hill to the gas phase. Replace the rubber tubing from both ports with serum rubber stoppers. Seal the contact of the stopper with the glass using silicone rubber adhesive. Take gas samples at the beginning and at the end of the experiment.
4. Separate the root, submerged shoot, and unsubmerged shoot from the rice plant and analyze N content and ^{15}N abundance.

c. Comment This method was used by Gowda and Watanabe (1985) to examine the effect of H_2 on dinitrogen fixation associated with rice roots and the basal portion of the shoot. After a 60-hr exposure to 84–63 atom% excess of N_2, the atom% excess of the root became 0.18–0.12. If a mass spectrometer is used, exposure time can be shortened. Barraquio *et al.* (1986) showed that excised root, including the basal portion of the shoot, could give similar ARA values similar to those given by intact plants.

3. $^{15}N_2$ incorporation associated with deepwater rice

Deepwater rice elongates the stem in the floodwater and, thus, accumulates a considerable biomass. Kulasooriya *et al.* (1981) reported the presence of epiphytic cyanobacteria and their photodependent nitrogen fixation on the root, leaf sheath, and, to a lesser extent, culm of the deepwater rice, which are under water. Because the portions active in nitrogen fixation are under water, only the submerged portions are exposed to $^{15}N_2$ (Watanabe and Ventura, 1982).

a. Materials required

1. Deepwater rice grown in pots and placed in a deepwater tank or in the deepwater area.
2. The assay chamber, as shown in Fig. 8.

b. Procedure Enclose the entire submerged portion plus about 15 cm of the aerial part of the rice culm in a plastic bag and seal with modeling clay. Fill the plastic bag completely with floodwater and submerge the assay chamber in the water. Introduce the labeled N_2 gas (1 liter of 90 atom% excess N_2 and 1 liter of ambient air) into assay chamber by displacing water from the inside. To promote dissolution of the labeled gas in the water, circulate the air through the assay chamber. Disconnect the air pump, and close the gas inlet and outlet. Take a gas sample for ^{15}N content determination. During assay, renew the labeled gas, if needed. Control plants without exposure to $^{15}N_2$ should be placed nearby. After exposure, divide the plants into various parts for ^{15}N content analysis.

c. Comment The assay chamber (1 m long) may include 70 liters of water, and the water contains 13.4 mg N liter^{-1} at 30°C at 80 kPa of N_2. The quantity of N_2 solubilized in water is far larger than the nitrogen fixed. Watanabe and Ventura (1982) showed 8 mg N fixation per plant in a 9-day period.

C. A ^{15}N Dilution Method for Estimating the Contribution of Nitrogen Fixation in *Azolla*

The *Azolla–Anabaena* system accumulates as much as 5 kg N ha^{-1}/day or 100 kg N ha^{-1}/mo. Because of its high nitrogen fixation rate, total N analysis is easily used to estimate the nitrogen gain of *Azolla* grown in flooded wetland or canals. However, to determine its nitrogen-fixing activity, the contribution of atmospheric N_2 to N gains in *Azolla* should be known (Watanabe *et al.*, 1991).

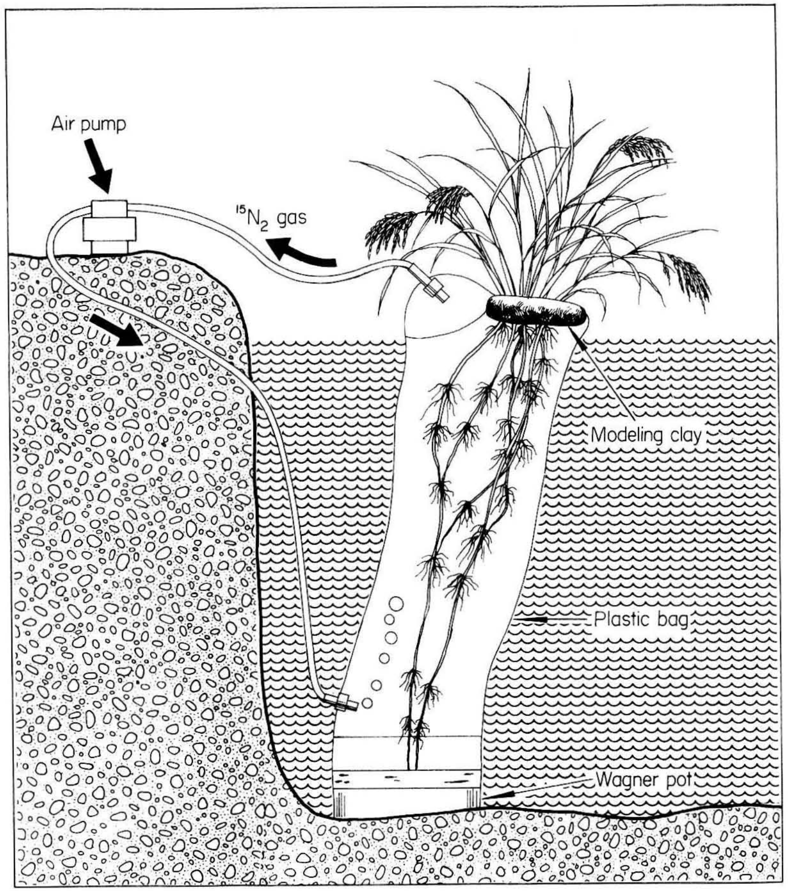

Figure 8 Incubation of deepwater rice with $^{15}N_2$ gas. [Reproduced, with permission, from Watanabe, I., and Ventura, W. (1982). Nitrogen fixation associated with deepwater rice. *Soil Sci. Plant Nutr.* **28**, 535–558.]

1. Materials Required

1. *Lemna minor* or *Anabaena*-free *Azolla* corresponding to the *Azolla* species used in the experiment.
2. Labeled $(NH_4)_2SO_4$ or urea (20 atom% excess), 4 g N m^{-2}.
3. A floating styrofoam frame, 20 × 20 cm in size.

2. Procedure

1. Prepare 1- × -1-m plots. Apply at least 4 g N m^{-2} as $(NH_4)_2SO_4$ or urea. If an emission spectrometer is used for isotope measurement, 20 atom% excess is needed. If a mass spectrometer is used, then 5 atom% excess will be adequate. Grow one or two crops of rice before the experiment. Preferably use plots that received the labeled substrate for a fertilizer experiment, if available.
2. Grow the reference plant and the *Azolla* in separate plots a few weeks before the experiment. Put two floating frames in a plot. Inoculate the reference plant (*L. minor*) into the two floating frames at the rate of 1 kg fresh weight m^{-2}. Inoculate the *Azolla* to be tested at the rate of 0.5–1.0 kg fresh weight m^{-2} outside the floating frames. Unless growth and nitrogen-fixing activity in P-deficient condition are desired, apply water-soluble phosphate at the rate of 0.13 g P m^{-2} every 3 days.
3. Grow both plants for 1 or 4 wk. The period of growth depends on the purpose of the experiment. If possible, take soil samples from 1-cm and 2–10-cm depths and floodwater before inoculating the Azolla and the reference plant and again at the end of the experiment.
4. Calculate the percentage of N derived from nitrogen fixation (%Ndfa) as follows.

$$\%\text{Ndfa} = \left(1 - \frac{\text{corrected atom\% excess of fixing plant}}{\text{corrected atom\% excess of reference plant}}\right) \times 100.$$

Corrected atom% = atom% excess at final biomass × factor
Factor is given as

$$\frac{\text{total N at harvest}}{\text{total N at harvest} - \text{total N of inoculated biomass}}$$

3. Comments

1. The substratum labeling technique can be used for labeling soil with ^{15}N. The ^{15}N isotope ratio in NH_4^+-N in floodwater decreases sharply after its addition to the floodwater due to the dilution by unlabeled mineralized NH_4^+. This rapid decline would create problems in applying the ^{15}N dilution principle. To avoid this rapid decline, a labeled compound should be added to a flooded rice soil long before the experiment. One crop or, preferably, two crops of rice are sufficient for the ^{15}N isotope ratio in mineralizable soil N to be stabilized. Kumarasinghe *et al.* (1985) applied 1–10 or 25 kg N ha^{-1} as ^{15}N-labeled urea once, just before inoculating plants to the plots. Scientists at the Fujian Academy of Agricultural Sciences in China (personal communication) used continual addition of labeled urea every 3 days to maintain the average ^{15}N isotope ratio of the available N in the floodwater throughout the experiment. The addition of labeled substrate to one or two crops of rice before the experiment has the advantage of a small change in isotope ratio in the NH_4^+-N in the floodwater.

An experiment at International Rice Research Institute (IRRI) (Watanabe *et al.*, 1991) showed that the atom% excess in NH_4^+-N at the 0–1-cm soil depth was 1.32 at the beginning of the experiment (after one crop of rice) and 1.62 at the end.

2. Kumarasinghe *et al.* (1985) used *Lemna* and *Salvinia*. In Fuzhou, *Anabaena*-free *Azolla* was used as a reference plant. Although *Anabaena*-free *Azolla* seems to be an ideal reference plant, very poor growth of this plant was observed under field conditions (Watanabe *et al.*, 1991). Unless *Salvinia* is grown near the experimental area, the use of this plant is discouraged because it is a very noxious weed.

3. The experiment at IRRI using soil rich in available P and *Lemna* as a reference plant showed that 80% of the N in *A. microphylla*, *A. filiculoides*, and *A. pinnata* was derived from the atmosphere. The %Ndfa of Azolla grown on the same soil, estimated by natural ^{15}N abundance method with *Lemna* or *Anabaena*-free *Azolla* as reference plants, was 73–78. These results show a similarity in the %Ndfa estimated by the two methods (Watanabe *et al.*, 1991).

4. The correlation of atom% excess of the harvested biomass is needed when the carryover of unlabeled N from the inoculated biomass is not negligible.

IV. AQUATIC ECOSYSTEMS

A. Incubation of Water Samples with ^{15}N

This method uses a gas introduction apparatus similar to that used by Neess *et al.* (1962) and is suitable for the analysis of ^{15}N by ratio mass spectrometry.

1. Materials Required

1. Van Dorn bottle for water sampling.
2. Glass bottles for incubation of water samples (1000, 500, or 250 ml, depending on the N content of the water).
3. Gas inlet system, as shown in Fig. 1.

1. Procedure

1. Collect water samples from the required depths in a water column using Van Dorn bottles. Transfer some of the Van Dorn sample into a plastic bottle or any other nontoxic sampling bottle.

2. Keep the samples, until they are exposed to $^{15}N_2$, under situations simulating *in situ* light and temperature conditions.

3. Transfer the water sample to the incubation chamber (Fig. 1a) of 1000, 500, or 250 ml, retaining a gas phase of 10% of the chamber volume. A volume is chosen to give at least 40 μg N in the water sample.

4. After the incubation chamber is connected to the apparatus (as shown in Fig. 1), purge a gas mixture (20% O_2, 80% Ar) through the incubation chamber for 20 min while keeping the pressure at the outlet from the chamber at 80 kPa. The required period of gas purging may be determined by measuring the gas composition at the outlet.

5. Close the two stopcocks of the incubation chamber and evacuate the gas inlet system. Transfer $^{15}N_2$ gas from the reservoir into the gas inlet system. The preparation and purification of the labeled gas are described in Section II.A.

6. Introduce $^{15}N_2$ gas into the incubation chamber by slightly increasing the gas pressure above ambient air pressure. The final composition of gas in the incubation chamber should be 20% $^{15}N_2$, 20% O_2, and 60% He or Ar (v/v).

7. After disconnecting the incubation chamber from the gas inlet system, shake the bottle by hand or by a wrist-action shaker to equilibrate the air and liquid phases.

8. Incubate for >1 hr in the laboratory under light or dark or at the sampling depth of the water column.

9. Stop the reaction by adding a small quantity of concentrated H_2SO_4 and reduce the volume of water under vacuum to accommodate the sample liquid in a 200-ml Kjeldahl digestion flask.

B. Incubation of Cyanobacteria with $^{15}N_2$

Cyanobacteria are major agents of N_2 fixation in euphotic layers of aquatic ecosystems. Some of them are so fragile that great caution is needed in sampling and incubation.

1. Materials Required

In place of a large volume incubation chamber, as shown in Fig. 1, use a 20–50-ml glass flask with serum stopper and aluminum cap (Fig. 1f).

2. Procedure

1. Take samples by appropriate water sampler or plankton net. Benthic cyanobacteria in bloom (epibenthic) can be picked up by hand.

2. Place samples in an incubation flask (10–25 ml) and close with a serum stopper. Cover the stopper with an aluminum cover. Connect the incubation flask to the hypodermic needle of the gas inlet system (Fig. 1f) and repeat evacuation and flushing with a gas mixture (80% He or Ar, 20% O_2, 0.03% CO_2). Start the reaction by introducing $^{15}N_2$ at 80 kPa.

3. Terminate the incubation by adding saturated mercuric chloride (final concentration of 0.1 m*M*).

3. Comments

1. Great variation of biomass and activity in space and time is a problem. This can be solved by collecting a large volume of water sample or running many trials in the season. Although most cyanobacterial nitrogen fixation is higher in the day than at night, some unicellular forms show dark nitrogen fixation. It is preferable, therefore, to conduct experiments to see the diurnal variations. These variations have been observed in freshwater (Horne, 1975) and marine environments (Saino and Hattori, 1978).
2. Pelagic cyanobacteria such as *Trichodesmium thiebautii* are fragile. When kept at highly crowded cell densities after sampling by net, cell constituents such as phycoerythrin are liberated from the cells and nitrogen-fixing activity is lost.
3. When the atom% of N at a natural-abundance level is measured by ratio mass spectrometry, the $\partial^{15}N$ value in the sample without the exposure to $^{15}N_2$ gas should be exactly determined. In aquatic systems, $\partial^{15}N$ values of organisms vary from −2 to 6‰. Even in samples collected by casting a plankton net, a variation in several ‰ is found. In this case, a sufficient number of controls is required.
4. A parallel run of ARA is recommended to assess the variability of the data.
5. The use of a syringe in place of a flask greatly reduces the time required for introducing the $^{15}N_2$ gas. Take each sample into a 40-ml syringe. Introduce the gas mixture (containing labeled N_2 gas up to 80 kPa) into the syringe; shake the syringe to solubilize the labeled gas in the liquid phase, and incubate *in situ*. After incubation, take the gas phase to determine the ^{15}N isotope ratio in the gas phase of the syringe. The $^{15}N/^{14}N$ ratio of the dissolved N_2 gas is almost the same as that of the gas phase.

C. Incubation of Sediments and Rhizosphere Samples with $^{15}N_2$

1. Procedure

1. Take cores (1.5 cm in diameter and 5.0 cm long) of sediment and place the sediment in a 125-ml Erlenmeyer flask.
2. Fully immerse the rhizome-root sample (1 g fresh weight) in 10 ml of habitat water in a 25-ml flask (Patriquin and Knowles, 1972; Zuberer and Silver, 1978).
3. Close the flasks with serum stoppers, evacuate, and flush with aerobic ($^{15}N_2$ + Ar + O_2 + CO_2) or anaerobic ($^{15}N_2$ + Ar + CO_2) gas mixture. Selection of the gas mixture depends on the redox condition of the sites.

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7

Nitrification and Denitrification

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I. DEFINITION OF PATHWAYS AND IDENTIFICATION OF PRODUCTS

A. Nitrification

Nitrification is the oxidation of ammonium (NH_4^+) to nitrate (NO_3^-). In soils, the process is mediated primarily by the autotrophic bacteria *Nitrosomonas* and *Nitrobacter*. *Nitrosomonas* oxidizes NH_4^+ to nitrate (NO_2^-), and *Nitrobacter* completes the oxidation to NO_3^- (Focht and Verstraete, 1977). The *Nitrosomonas* oxidative process may be represented by the following sequence:

$$2\ NH_4^+ \rightarrow 2\ NH_2OH \rightarrow$$

$$2\ (NOH) \rightarrow 2\ NO \rightarrow 2\ NO_2^-$$

$$\searrow N_2O \swarrow$$

The gaseous products NO and N_2O are both produced as a result of NH_4^+ oxidation. The exact mechanisms of production of these gases are not certain at this time. Kaplan *et al.* (1988) showed that in a tropical forest significant quantities of NO are produced and released to the atmosphere during nitrification, generally exceeding N_2O. Blackmer *et al.* (1980) showed that N_2O is produced as a result of nitrification (the exact reaction sequence is not known), and Poth and Focht (1985) showed that certain nitrifying microorganisms generate N_2O from the reduction of NO_2^-, which they produced under O_2-limiting conditions.

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Nitrosomonas and *Nitrobacter* oxidize about 35 and 100 N atoms, respectively, for each molecule of CO_2 assimilated. Both organisms are gram-negative, aerobic, chemoautotrophic rods (Focht and Verstraete, 1977). Nitrification is controlled primarily by NH_4^+ and O_2 concentrations. Oxygen supply is moderated by soil moisture, and in the normal soil moisture range the effect of soil water content on N transformations probably reflects its effect on O_2 diffusion. The nitrifiers are active over a wide range of temperatures (2–40°C) and the optimum pH for the organisms lies between 7 and 9.

Rates of nitrification can be measured by mass balance techniques, but considerable nitrate turnover can occur in soils (Schimel, 1986). This requires the use of an isotope technique to measure gross nitrate production (Schimel, 1987) (see also Chapters 8 and 9 on measuring gross mineralization).

B. Denitrification

Denitrification is defined as the dissimilatory reduction of oxides of nitrogen to produce N_2O and N_2 by a taxonomically diverse group of aerobic bacteria, which synthesize a series of reductases enabling them to utilize successively more reduced N oxides as electron acceptors in the absence of O_2 (Knowles, 1981). The most abundant denitrifiers are heterotrophs, which require sources of electron-reducing equivalents contained in available organic matter. The generally recognized reductive sequence is $2\ NO_3^- \rightarrow 2\ NO_2^- \rightarrow 2\ (NO) \rightarrow N_2O \rightarrow N_2$. Some controversy still exists as to the obligatory nature of NO intermediacy. Aerssens *et al.* (1986) indicated that the reduction of NO_2^- to N_2O is accomplished by one enzyme and that NO is only a side-product resulting from decomposition of NO^+. Conversely, Goretski and Hollocher (1988) suggested that NO is a freely diffusible intermediate between NO_2^- and N_2O.

The soil factors that most strongly influence this reductive sequence are O_2, soil water content, NO_3^- concentration, pH, temperature, and organic carbon. The reductive enzymes are repressed by O_2 but not by NH_4^+. Nitrous oxide reductase appears more sensitive to O_2 than either NO_3^- or NO_2^- reductase. Therefore, N_2 production predominates in more anoxic sites and N_2O production may be greater in more aerobic conditions. Because of the heterogeneity of the factors controlling denitrification, the spatial and temporal variability of this process in soil is large.

Efforts to directly measure denitrification in soils are generally centered around quantifying N_2O and N_2 production in and evolution from the soil system. Other indirect studies based on the disappearance of NO_3^- from the soil have been used in denitrification studies. ^{15}N is readily used in both types of studies, and we describe its function in a number of examples.

II. NITRIFICATION EXPERIMENTS

A. Measuring Nitrate Production Using ^{15}N Dilution

Isotope mass balance (Schimel, 1986) approaches have been employed as well as simple (Schimel, 1987) and complex (Myrold and Tiedje, 1986) isotope dilution models. Measurement of nitrification rates using pool dilution requires both appropriate field methods and mathematical treatment of the data.

Three considerations are important in designing field experiments for $^{15}NO_3^-$ measurements. First, mass balance must be preserved. That is, losses of NO_3^- by leaching or lateral movement can confound calculations of NO_3^- turnover. Thus, enclosed plots or laboratory incubations are preferred (Clark, 1977; Schimel, 1986, 1987).

Second, recycling of N causes difficulty in analyzing dilution experiments. That is, if NO_3^- is immobilized, mineralized to NH_4^+, and renitrified, this causes isotope ratio changes that can be very difficult to analyze, especially in experiments that do not reach steady state (Kirkham and Bartholomew, 1954; Shipley and Clark, 1972; Schimel, 1986). If experimental assays are kept brief so that recycling is not quantitatively significant, subsequent analysis is greatly simplified. Field assays may range from 1 to a few days. Laboratory assays may be hours to days. The exact duration will vary with the level of microbial activity in the soil. Length of incubation should be inversely proportional to rate of reaction. This will both preserve sensitivity and minimize the effects of recycling. Sensitivity will increase as the amount of isotope converted to organic or gaseous forms increases with time, providing a better signal to noise ratio.

Third, distribution of NO_3^- in the soil should be both macroscopically and microscopically homogeneous. Macroscopic homogeneity is needed because the entire volume of soil incubated will not be analyzed. One subsample would be used for total N analysis, with another reserved for inorganic N. Homogeneity may be achieved by careful sprinkling with dilute isotope or by injection in a grid at prescribed depth (Schimel, 1987; Schimel and Firestone, 1989).

Microscopic heterogeneity is a more difficult problem. Soils are not homogeneous bodies and contain "microsites" with varying organic matter and oxygen concentrations. Anaerobic microsites in a soil that has bulk aerobic conditions can result in significant reductive N metabolism. If $^{15}NO_3^-$ does not mix homogeneously with the native soil NO_3^-, the heavy isotope may undergo rates of conversion to gaseous or organic forms that are not representative of the native NO_3^-. To avoid this problem, careful injection or mixing is required, and attention should be paid to the moisture status of the soil. Obviously, mixing will be more uniform in a wet than in a dry soil. No complete solution to the mixing problem has been devised, and researchers should be attentive to this potential problem.

B. Preparation of NO_3^- Samples for ^{15}N Analysis

Nitrate must be extracted from soil and converted to a solid NH_4^+ species for mass spectrometric analyses. A number of methods exist for carrying out the extraction, purification, and reaction to NH_4^+ steps of this process; we describe a simple and well-tested approach and mention a few innovative techniques that are in development as a consequence of improved mass spectrometer systems (see also Chapter 2).

First, inorganic N must be extracted from soil. This is normally done using a 2 *M* solution of KCl in water, prepared with deionized N-free water. KCl solution is added to soils in a 5 : 1 ratio (volume : mass), shaken for 0.5–2 hr. The shaken sample should then be filtered through a prerinsed filter, and the filtered solution retained for subsequent analysis.

At this point, the samples should be analyzed for inorganic N species: NO_3^- and NH_4^+. This may be accomplished by any number of standard chemical methods, descriptions of which are outside the scope of this chapter. The reader is referred to Keeney and Nelson (1982). This analysis is essential to preparing the sample for further analysis because a minimum quantity (10–500 μg) of N is required by mass spectrometers for satisfactory analysis. Sufficient N must be prepared for mass spectrometric analysis, and this influences the quantity prepared in the following steps. The exact quantity of N required must be ascertained for the mass spectrometer that will be used. The quantity is calculated from volume required (ml) = mass of N required (μg)/([NO_3–N] in KCl solution (μg/ml).

Equipment required for the following procedure includes an aluminum heating block (block digester) and diffusion tubes. Diffusion tubes are prepared as in O'Deen and Porter (1979). In brief, they are 40-cm-tall tubes that fit in the block digester. The upper part should be closed with a gas-tight screw cap and three dimples are pressed into the upper part of the tube to support a small vial (2 ml) just below the screw cap. Prior to analyses, all glassware should be cleaned with hot tap water, rinsed with 0.1 *M* $(NH_4)_2SO_4$ prepared with natural-abundance salt (to remove residual enriched or depleted N), soaked in an acid bath, rinsed with deionized water, and dried.

To conduct the conversion–diffusion, place a quantity of KCl solution containing the amount of N required but not more than 35 ml in a diffusion tube. If more than 35 ml is required, repeated diffusions into the same trap must be carried out. The solution must then be made basic to drive off the NH_4^+ present. To accomplish this, 13 *M* NaOH is added. The NH_3 may be trapped or allowed to vent but must be removed prior to NO_3^- analysis. After addition of the NaOH, the tubes should be sealed and heated to 105°C for 48 hr. If desired, a vial containing 2 ml of 1 *M* HCl may be added to trap the NH_3 evolved for isotopic analysis. Devarda's alloy should then be added to the solution (Bremner and Keeney, 1966) to convert the NO_3^- to NH_4^+. Also add a new vial containing 1 *M* HCl. The solution should be allowed to react for 18 hr at 80°C. Either close the tube with a rubber stopper pierced by a small glass tube or open the tube periodically. This serves to vent H_2 gas produced by the Devarda's alloy and prevents the tubes from exploding and, when performed carefully, results in no detectable NH_3 loss. Then seal the tubes and raise the temperature to 105°C and diffuse as described earlier for NH_3.

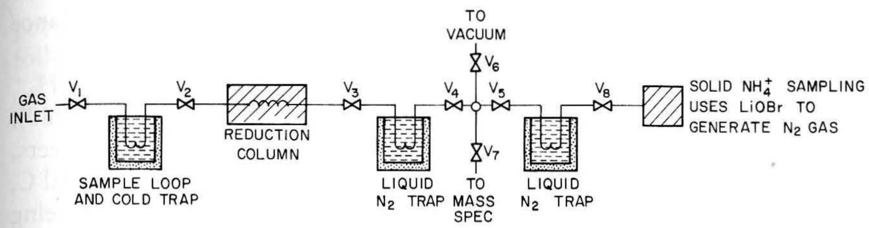

Figure 1 Simple mass spectrometer gas and solid ammonium sampling inlet system. Sample loop is 2–5 ml volume to be used with or without a cold trap; reduction column is heated to 250–300°C. V, vacuum-tight valve.

After diffusion for either N species, the small vials (shell vials) should be dried at low heat (<100°C). The dried salt is then ready for mass spectrometric analysis. Porter and O'Deen (1977) describe a N_2 gas preparation system that is attached directly to the mass spectrometer inlet. The schematic drawing of a version of this system is provided on the right side of Fig. 1. Dinitrogen is generated from reaction of NH_4Cl with LiOBr in the shell vial; water vapor and other impurities are removed by passing the gas produced through a liquid N_2 trap before the gases enter the istotope ratio mass spectrometer (IRMS) inlet (Fig. 1).

A few precautions should be observed using this method. The diffusion process strongly discriminates between isotopes, so that the light isotope diffuses out of solution first. Quantitative recovery of the N is required to prevent error. Samples containing known amounts of N should be included as controls over recovery rate. For enriched samples (>1 atom% excess), recoveries of >95% appear adequate. The diffusion method is not suitable for preparation of samples for natural abundance analyses. Losses or retention of N too small to detect by measuring the quantity recovered can cause noticeable error in estimates of near-natural abundance N. In addition to the mass balance check, analysis of samples with known ^{15}N abundance should be conducted.

Several alternative approaches exist for separation of NO_3^- and NH_4^+ from solution. Distillation may be used, as described in Reeder *et al.* (1980). Recently, mass spectrometers have been interfaced to elemental analyzers, the analyzers using combustion-gas chromatography to determine total C, N, S, and H. Modifications of the preceding diffusion procedure are being developed to trap NH_3 in solid combustible forms. The solid trap may be burned in the elemental analyzer and the gases fed into a mass spectrometer (Brooks *et al.*, 1989; Burke *et al.*, 1990).

C. Pool Dilution Models

Rates of nitrification may be calculated in short-term experiments using the equations of Kirkham and Bartholomew (1954) and Schimel (1986, 1987). In this model, nitrification is calculated as follows: gross NO_3^- production = (net NO_3^- appearance)$*$log [(initial $^{15}NO_3^-$$*$final $^{14+15}NO_3^-$)/(initial $^{14+15}NO_3^-$$*$final $^{15}NO_3^-$)]/log (final $^{14+15}NO_3^-$/initial $^{14+15}NO_3^-$). Calculations should be done in units of mass. Net nitrate production is final − initial NO_3^-. Units of time may be seconds or days, depending on the length of incubation, and must be related to the interval to which the calculation applies. Final units are micrograms (μg N)$^{-1}$ days^{-1}. This calculation only applies if NO_3^- production is greater than NO_3^- uptake, or net NO_3^- production is >0. Other calculations apply when consumption exceeds production and may be derived from equations in Kirkham and Bartholomew (1954).

More complex models have been developed for the simultaneous estimation of mineralization, immobilization, and denitrification (Van Cleve and White, 1980; Myrold and Tiedje, 1986). These models use zero- and first-order kinetic expressions, with parameters estimated with nonlinear parameter estimation. Myrold and Tiedje's (1986) model allows simultaneous estimation of multiple N-cycling parameters, even under nonsteady-state conditions. Application of such multiple pool models is complex and outside the bounds of the present discussion. The reader is referred to the literature for a complete description. This type of model is recommended for complex experiments.

D. Example Nitrification Experiment

Here we estimate gross nitrification using pool dilution and the Kirkham and Bartholomew (1954) calculations (Schimel, 1987). Assume that 5 μg ^{15}N-NO_3^- are added to a volume of soil. The N should be injected carefully (in a field experiment) or well mixed (in the lab). Initial $^{14}NO_3^-$ must be determined as well. After 1 day of incubation, the sample is processed and final NO_3^- determined. The data for the experiment are as follows:

Day	$^{15}NO_3^-$ N (μg)	Total NO_3^- (μg)
1	5.0	10.0
2	1.5	12.0

From these data, we can calculate net and total nitrification. Net nitrification is simply final − initial NO_3^-, or 12 − 10 = 2. Total nitrification is calculated according to Kirkham and Bartholomew's equations given previously. Note that net nitrification is a rate, defined as the net change in NO_3^- between the initial and final measurements. The final calculation is then a rate with the units of time defined by the length of the incubation.

In this example, total nitrification = (2)$*$log [(5$*$12)/(10$*$1.5)]/log (12/10) = 15.24 μg NO_3^- N days^{-1}. This would indicate very active turnover of nitrate because total nitrification was 15.24 and net was 2. This implies that 13.24 μg of NO_3^--N was converted to organic matter (immobilized), denitrified, or converted to NH_4^+ in 1 day. Such data are obviously a very valuable complement to direct measures of these other fluxes. Techniques for measuring the other sinks of inorganic N are discussed in Chapter 4, this volume.

III. ANALYSIS OF ^{15}N-CONTAINING GASEOUS NITRIFICATION AND DENITRIFICATION PRODUCTS

The most commonly used methods for NO and N_2O analyses are chemoluminesence techniques and "hot" electron capture gas chromatography (ECD-GC), respectively. It is possible to utilize ^{15}N in analysis of these two N gases. Mulvaney and Kurtz (1982) discussed N_2O analysis in detail and we briefly describe their method later. Because of the difficulty in separating NO and N_2O cryogenically, a GC separation procedure may be used. The gases can be individually cryogenically trapped from the GC effluent and the ^{15}N isotopic abundance determined by isotope ratio mass spectrometer (IRMS) analyses using the gas sampling inlet systems described later for N_2O. A GC-quadrapole mass spectrometer can be used to separate NO and N_2O and concurrently determine the ^{15}N content of the separate gases. We will not discuss this technique further but refer the reader to Focht (1978) and Poth and Focht (1985).

A. Mass Spectrometer Inlet System

Dinitrogen ^{15}N content is readily measured by IRMS and is the technique described herein. To perform the analyses, however, gases must be first purified. The studies described later, which demonstrate the techniques, were conducted in an air atmosphere; therefore, the contaminants O_2, CO_2, and CO, which interfere with N isotope analysis, must be removed before the N_2 gas is introduced into the IRMS ionization source. It is most important to remove O_2 because it reacts with N_2 in the ion source to form NO, *m/e* 30, and decreases the IRMS source filament life. Carbon dioxide is partially fragmented to CO in the ion source, which produces *m/e* 28, 29, and 30. Nitrous oxide is partially decomposed to NO in the ion source, which can contribute to a false *m/e* 30 reading.

To eliminate contamination and to ensure that pure N_2 is introduced into the IRMS, a gas sampling inlet system is required. The system can be relatively simple (Fig. 1) or more complex as shown by Mulvaney and Kurtz (1982). The simple system facilitates gas purification and quantitative analysis of N gases. The simple inlet consists of a series of stainless-steel components, a 2–5 ml sample loop ($\frac{1}{8}$", OD tubing), an O_2 and N_2O scrubber, and a liquid N_2 cold trap (Fig. 1), each connected by a vacuum-tight valve to permit isolating each portion of the inlet. The inlet is arranged to permit introducing a gas sample directly from a syringe fitted with a one-way luer stopcock into the sample loop. The loop can be immersed in a cryogen (liquid N_2 or dry ice-cooled 2-pentanol) to remove water vapor, CO_2, or N_2O, depending on the desired analysis. From the sample loop, the gas passes into the O_2 scrubber. This scrubber is made by packing a tube with about 10 g of reduced copper material (L. C. Company, 619 Estes Avenue, Schaumburg, Illinois 60193).[1] This scrubber can be operated at room temperature but efficiency is greater if an elevated temperature is used. At a temperature of 250–300°C, the trap effectively removes O_2 and reduces N_2O and NO to N_2 (Mulvaney and Kurtz, 1982). About 30 sec after introducing the sample into the O_2 scrubber, it is passed through the high-efficiency liquid N_2 trap to remove CO_2 and H_2O. The purified sample N_2 is then passed into the IRMS inlet. Both sample and reference gases are introduced into the IRMS through the gas inlet so that they are directly comparable. If a dual inlet IRMS is available, a time zero field gas sample is introduced into the reference side of the IRMS inlet through the sample inlet to serve as reference gas. For laboratory studies, the atmosphere used in incubation flasks, air or N_2, is used as reference gas. If a single inlet instrument is used, then samples and reference gases must be run sequentially.

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[1] Tradenames and company names are included as a matter of convenience to the reader, and such inclusion does not constitute any preferential endorsement by the U.S. Department of Agriculture of products named over similar products available on the market.

B. Calculations

The ^{15}N method that we use to quantify total denitrification in a field or laboratory system was first proposed by Hauck *et al.* (1958). Hauck and Bouldin (1961) described the method further but its applicability did not become evident until Siegel *et al.* (1982) revitalized the technique and modified the calculations. Further modifications in calculations are described by Mulvaney (1984) and Mulvaney and Boast (1986). The method involves applying highly ^{15}N-enriched fertilizer to the soil (>20 atom% ^{15}N) and then using a chamber to cover the ^{15}N-fertilized plot to isolate the atmosphere above the soil for a designated time. This permits determining the rate of change of ^{15}N atoms (N gases) in the chamber atmosphere over time. Note that the calculation revisions made by Mulvaney and Boast (1986) provide more accurate estimates if the NO_3^- is <20 atom% ^{15}N. The calculations utilize the fact that the soil N gases (principally N_2 under usual denitrifying conditions) that evolve into the chamber headspace containing normal air do not randomly mix isotopically with the N gases in the chamber. Utilizing this nonrandom ^{15}N distribution, the method permits calculation of the amount of N gas evolved not only from the added ^{15}N-enriched fertilizer but also from soil N, when denitrification occurs. Because the ^{15}N mole fraction of the NO_3^- in the soil that serves as the N-gas source is calculated directly from mass spectral data [*m/e* 29/28 and 30/28 ratios or 29/28 and 30/28 + 29 if only a double collector IRMS is available (Mulvaney and Kurtz, 1982)], it is not necessary to disturb the soil in the plot during the course of the experiment. The soil NO_3^- ^{15}N content must be known to calculate the total amount of N_2 evolved from the site at any given time.

Rather than describing the equation derivation, we refer you to the manuscripts by Siegel *et al.* (1982), Mulvaney (1984), and Mulvaney and Boast (1986). Table I summarizes the basic simple version of equations used to calculate the total N-gas flux from the soil using a triple collector IRMS. The technique is applicable to laboratory incubation flasks or field studies, and total N gas production is estimated, not merely the N gases produced from the added nitrogen.

One must also recognize that the equations used require a number of assumptions. The first is that the total amount of $^{28}N_2$ inside the gas collection chamber or incubation flask does not change during the sample collection period. Siegel *et al.* (1982) noted that providing the chamber $^{28}N_2$ is 100 times or more greater than the amount of gas produced, the calculations are correct. The equations of Mulvaney and Boast (1986) do not rely on this assumption, however. The second and major assumption is that the ^{15}N label of the NO_3^- in the soil is uniform. We assume that nitrate formed from the ^{15}N-labeled fertilizer added to the soil mixes

Table I
Equations Used to Calculate N-Gas Emissions from Mass Spectrometric Isotope Ratio Measurements of Gas Samples

1. $\Delta r = (^{29}N_2/^{28}N_2)_{\text{sample}} - (^{29}N_2/^{28}N_2)_{\text{reference}}$
2. $r' = (^{30}N_2/^{28}N_2)_{\text{sample}} - (^{30}N_2/^{28}N_2)_{\text{reference}}$
3. Sample = air sample from collection chamber at some time, t, after installing chamber
4. Reference = air sample from field, i.e., normal air sample taken from the chamber immediately after installation
5. 29/28 and 30/28 are ion current ratios determined by the mass spectrometer.
6. $^{15}X_N$ = mole fraction of ^{15}N in the soil NO_3^- pool = $2(\Delta r'/\Delta r)/(1 + (2(\Delta r'/\Delta r)))$
7. d = fraction of total N gas in the gas collection chamber attributable to denitrification $= \Delta r'/(^{15}X_N)^2$
8. Total N gas evolved from the soil into the collection chamber = total N_2 in the chamber volume$^*d$
9. N_2 flux $= \Delta C/(A^*\Delta t)$,
   where A is soil surface area covered by chamber,
   Δt is time that the chamber covered the soil, and
   ΔC is the change in the amount of $^{30}N_2$ and $^{29}N_2$ in the chamber during time t.
   $(\text{total } N_2 \text{ in chamber}^*d)_{t_1} - (\text{total } N_2 \text{ in chamber}^*d)_{t_0}$

uniformly with the unlabeled NO_3^- already in the soil or with unlabeled NO_3^- formed from organic N mineralization. Nonuniform mixing of the ^{15}N label may cause underestimation of the total N denitrified in some cases. However, Mulvaney (1988) indicates that accurate N-gas production estimates can be made when the soil NO_3^- pool is not uniformly labeled. An example calculation is shown in Table II.

C. N_2O Analysis Using ^{15}N

The amount of N_2O evolved from a ^{15}N-fertilized soil can also be quantified by IRMS analyses (Mulvaney and Kurtz, 1982). Using the inlet shown in Fig. 1, an air sample collected from an incubation flask or soil cover enclosure is introduced into the gas sampling inlet of the IRMS. The inlet sampling loop is immersed in liquid N to freeze out N_2O and the N_2 is analyzed. After N_2 analysis, the inlet system is evacuated and a known volume of unlabeled N_2 is added to the sample loop. The sample loop is warmed to gasify the frozen compounds and the N_2O and N_2 are mixed and then passed through the O_2 scrubber. The N_2 produced is then analyzed as described earlier and the same calculation process is used, except that the quantity of N_2O is equal to d times the amount of N_2 dilution gas used. The procedure requires that the amount of dilutent gas exceed the amount of N_2O in the sample by 100 times if the equations in Table I are used. This assumption is not needed for the Mulvaney and Boast (1986) equations. The gas sampling inlet described in Mulvaney and Kurtz (1982) is probably better suited for N_2O analysis because the added N_2 and residual N_2O are thoroughly mixed by rapidly circulating the gas in the inlet with bellows pumps.

Table II
Example of N-Gas Flux Calculation, Using Data from Field Case Study 3

	Isotope ratios			
	Sample gas 4 hr Plot 1		Reference gas 0 hr Plot 1	
Replicate	29/28	30/28	29/28	30/28
1	0.0073465	0.0000751	0.0072948	0.0000331
2	0.0073485	0.0000751	0.0073089	0.0000331
3	0.0073451	0.0000749	0.0073010	0.0000328
Mean	0.0073467	0.0000747	0.0073016	0.0000329

$$\Delta r = 0.0073467 - 0.0073106 = 0.0000451$$

$$\Delta r' = 0.0000747 - 0.0000329 = 0.0000418$$

$$^{15}X_N = 0.651$$

$$d = 0.0000418/(0.651)^2 = 0.0000986$$

To calculate the amount of N_2 inside the chamber (7775 ml), assume that air is 78% N_2; correct to standard temperature and pressure (STP), 273 K and 760 mm Hg; assume that the temperature was 20°C (293 K) and the atmospheric pressure was 760 mm Hg. With these assumptions, then calculate that there was a total of 5651 ml N_2 inside the chamber. To calculate the mass of this N_2, we know that 1 mole of gas at STP occupies 22,400 ml; then 5651 ml of gas is 0.252 moles or 7.064 g of N_2. The amount of N gas evolved from the soil into the gas chamber is therefore:

$$\text{total N} = 0.00009863 \times 7.064\text{ g} = 697\ \mu\text{g N}$$

$$N_2 \text{ flux} = 697\ \mu\text{g N}/707\text{ cm}^2 \times 4\text{ hr} = 0.2465\ \mu\text{g N cm}^{-2}\text{ hr}^{-1}$$

On a hectare basis then 24.7 g N ha^{-1} hr^{-1} evolved from the soil.

IV. DENITRIFICATION EXPERIMENTS

A. Laboratory Case Studies

The preceding ^{15}N technique is readily applicable to laboratory denitrification studies. It is particularly advantageous because the studies can be performed under air or N_2 atmosphere. Using the simple version of the calculations requires that the incubation chamber headspace N_2 content be more than 100 times greater than the amount of $N_2 + N_2O$ produced during denitrification. We use two laboratory experiments to illustrate the applicability of the method and to show potential problems that may be encountered.

1. Lab Case Study 1

This study was performed (Haider *et al.*, 1983a,b) to observe the effect of acetylene on denitrification. In this study, 10 g soil (a clay loam containing 0.9% C, 0.16% N, pH 7.3) were placed in 125-ml serum bottles and 10 ml aqueous solution containing 2 mg KNO_3-N (99 atom% ^{15}N) was added. The bottles were immediately sealed with rubber serum caps and, when desired, acetone-free acetylene was injected to attain 10% (v/v). The bottles were incubated for 3, 6, and 12 hr and 1, 3, and 7 days at 25°C. After these incubation periods the gas headspace was analyzed for N_2O and CO_2 by GC (Mosier and Mack, 1980) and $N_2 + N_2O$ by IRMS, as described earlier. After gas analysis, the soil from each bottle was extracted with 50 ml 2 *M* KCl. The KCl extract was analyzed for $NO_3^- + NO_2^-$ and NH_4^+ by steam distillation and processed for ^{15}N analysis (Bremner and Keeney, 1966). An aliquot of the KCl-extracted soil was Kjeldahl-digested for total N and ^{15}N determination. Samples were analyzed for ^{15}N as described by Porter and O'Deen (1977) with a triple-collector IRMS. The techniques described in Section II.B are alternative methods for analyzing mineral N and ^{15}N content.

The short-term effect of acetylene on denitrification in a water-saturated soil with no exogenous carbon is shown in Fig. 2. The total $N_2 + N_2O$ production was significantly greater ($p < 0.025$) when the soil was exposed to acetylene, at each incubation period. After 1, 3, and 7 days (see Haider *et al.*, 1983a), significantly more NO_3^- was reduced and more $N_2 + N_2O$ produced in the presence of acetylene.

2. Lab Case Study 2

One of the potential limitations to the ^{15}N technique, particularly in field studies of both nitrification and denitrification, is that the method assumes that the NH_4^+ or NO_3^- in the soil undergoing transformation has a uniform ^{15}N distribution. The ^{15}N added is assumed to mix uniformly with the unlabeled NO_3^- so that the denitrifying microflora encounter a NO_3^- pool that has a uniform ^{15}N content. Unless systems are well mixed, it seems unlikely that this uniform ^{15}N-NO_3^- distribution can exist. Theoretically, this nonuniform distribution leads to underestimating the total amount of denitrification that occurs in a soil (Focht, 1985; Mulvaney, 1988). The following experiment (Mosier, unpublished) was performed to determine if evolution of N_2 into a common headspace from physically separated NO_3^- pools, containing different ^{15}N contents, underestimated total denitrification.

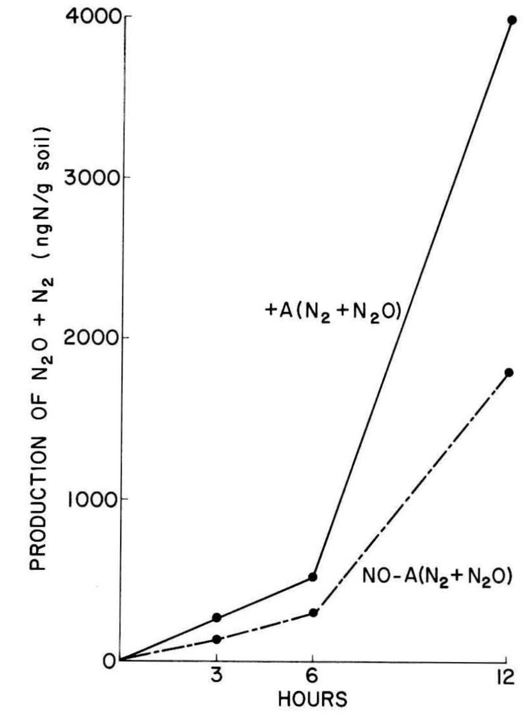

Figure 2 Effect of acetylene on short-time production of N_2O and N_2 from a NO_3^- amended, water-saturated soil under an air atmosphere.

One-pint preserving jars (568 ml) were used as incubation chambers. A gas sampling port was made in each rubber-gasketed lid by making a 6-mm-diameter hole with a cork borer and inserting a rubber serum stopper. Silicone caulking was used around the stopper to ensure an airtight seal. Inside each jar were placed three 35-ml liquid scintillation vials. One vial contained 2 ml 1 *M* NaOH to collect CO_2 and each of the other two vials contained 10 g soil (as described in Lab Case Study 1). To each vial of soil was added 1 or 2 ml KNO_3 solution, totaling 200 μg N/g soil. The ^{15}N enrichment of the NO_3^- varied between 0 and 70 atom% ^{15}N in different vials. The individual vials represented separate NO_3^- pools for which the ^{15}N enrichment was controlled. After the NO_3^- solution, 1 ml of glucose solution (15 mg glucose-C/ml) was added. Finally, 1.25 or 0.25 ml of distilled water was added to bring the final moisture content of the soil to 35%. A set of time zero samples was prepared and analyzed immediately to provide a zero sample basis for comparing total and ^{15}N values for each experiment. The ^{15}N-fertilized soils were then incubated at 25°C under an air atmosphere for 1–7 days. The atmosphere of half of the jars was amended with 5% (v/v) of acetone-free acetylene to block the conversion of N_2O to N_2. Adding acetylene allowed cross-comparison of the amount of N gas produced by two different methods. By measuring N_2O produced in the presence of acetylene by GC and the total N gas produced by IRMS, the two analyses should give the same result for gas produced, unless the ^{15}N method underestimated N-gas production.

After the designated times, soils were sampled in triplicate and analyzed for total N and ^{15}N. The remaining soil was extracted with 2 *M* KCl and the extract analyzed for $NO_3^- + NO_2^-$ and NH_4^+ and ^{15}N (see Section II.B).

Samples of the jar's gas phase were removed through the rubber septum in the jar lid using 3-ml polypropylene syringes, fitted with vacuum-tight stopcocks. Two samples were collected each time: 1.5 ml for IRMS analysis and 1.0 ml for N_2O analysis by GC. The total gas volume, accounting for vials, soil, and solution, was measured for each jar. Recall that the experiments were set up so that separate vials of soil were amended with NO_3^- having the same or different ^{15}N enrichments. The gases produced from each vial evolved into the jar atmosphere, where the gases mixed with the initial jar air atmosphere. Hauck and Bouldin (1961) showed that the N_2 molecules formed from the soil and from ^{15}N-NO_3^- have a distinct ^{15}N content and, although the N_2 molecules formed from the different NO_3^- pools physically mix in the gas phase, they do not mix atomically. Therefore the equilibrium reaction $^{15}N^{15}N + {}^{14}N^{14}N \leftrightarrow 2\ {}^{15}N^{14}N$ does not occur appreciably. If we compare N-gas production from soils in which two NO_3^- pools are present, one containing no ^{15}N enrichment and the other containing 70 atom% ^{15}N, using acetylene block and ^{15}N methods, we see that the total amount of N evolved, denitrified, is underestimated by the ^{15}N method. When acetylene was used to block N_2O reduction to N_2, 855 μg N was recovered compared to 380 μg total N measured in the same gas sample by IRMS (Table III). This demonstrated that when the NO_3^- pools were physically separated so that it was impossible for the NO_3^- pools to mix, and one of the pools had no ^{15}N enrichment, the ^{15}N method underestimated the amount of N gases produced as predicted from theory. When the NO_3^- pools were both either 30 or 50 atom% ^{15}N, the amount of N gas produced measured by both methods was the same throughout the 7-day incubation (Table III). In the cases where the NO_3^- pools were 30 and 50 atom% ^{15}N, both methods estimated the same amount of N-gas production ($p < 0.01$). These results are similar to those presented by Mulvaney (1988), which show that the technique accurately represents the amount of N gas produced during denitrification even when all nitrate pools do not have the same atom% ^{15}N labels, but each pool must have some ^{15}N label.

Table III
Effect of Varying the ^{15}N Content of Two Different NO_3^- Pools, with Acetylene Added, on the Production of N Gases

NO_3^- Atom% ^{15}N		Incubation Period (Days)					
		1		3		7	
Vial 1	Vial 2	GC[a]	MS[b]	GC	MS	GC	MS
			μg N				
30	30	68	96	870	870	1280	1280
50	50	100	190	560	620	650	690
30	50	100	190	860	890	960	940
70	0	215	165	570	320	855	380

[a] GC indicates N_2O measured by GC (acetylene block technique).

[b] MS indicates total N gas production ($N_2 + N_2O$) measured by mass spectrometry (^{15}N method).

B. Field Case Studies

The ^{15}N method described above for laboratory studies is also directly applicable to field denitrification studies. In any field study, the experimenter hopes to maintain the experimental unit in as near to normal field conditions as possible. Unfortunately, ^{15}N chemicals labeled with >20 atom% ^{15}N are expensive (more than \$25/g compound) and movement restrictions are needed. Therefore, most experiments are conducted in semiconfined units located in small plots using confining barriers to prohibit lateral movement and uptake by plants from outside the ^{15}N-fertilized soil. An additional nonnatural condition is that soil covers must be used to measure the relatively small instantaneous upward flux of N_2 or N_2O from the soil. Such chambers enclose a distinct volume of air above a known area of soil and prevent emanating N_2 and N_2O from mixing with the atmosphere outside the chamber. Potential problems associated with soil cover gas collection methods are discussed by Denmead (1979), Hutchinson and Mosier (1981), and Jury *et al.* (1982), and we will not discuss this important aspect further.

We should further note that the field ^{15}N method quantifies the flux of gas from the soil to the atmosphere above. It does not directly measure the instantaneous soil denitrification rate. Diffusion of gases through the soil is regulated by the soil pore space water content. The movement of a gas, N_2 for example, from its production site in the root zone to the atmosphere may require days [see Letey *et al.* (1980) for a discussion of N gas diffusion in the soil]. In flooded soils, N gases may remain entrapped in the soil water matrix for extended times (Lindau *et al.*, 1988). N-gas flux can be measured frequently over discrete time periods and these numbers accumulated to measure the total N gas produced as a result of nitrification and denitrification.

1. Gas Collection and Analyses

As already mentioned, analytical methods are not sufficiently sensitive to directly measure the small upward flux of N gases from the soil to the atmosphere. We typically used a closed cover technique described by Hutchinson and Mosier (1981). This cover is vented to the atmosphere so that the soil surface enclosed by the cover sees the same atmospheric pressure fluctuations as does the uncovered soil. The chamber is also thermally insulated so that the soil temperature does not appreciably increase during the time that the cover is in place. The concentrations of $^{30}N_2$ and $^{29}N_2$ or N_2O beneath the soil cover will increase whenever there is a flux out of the soil.

Immediately after installing the covers over a ^{15}N-treated soil plot, an initial (time zero) gas sample is withdrawn from the chamber. The sampling device we typically use is a polypropylene syringe fitted with a one-way, gas-tight stopcock. When possible, the samples are returned to the laboratory and analyzed within a few hours of collection. When immediate analysis is not possible, gas samples are stored in rubber-stoppered, freshly evacuated glass tubes (Vacutainers® are a commercial example). The transfer from the syringe to the storage tube is made by injecting 12 ml of gas into an 11-ml tube to produce a positive pressure to limit leakage into the vial. The stopper injection hole is resealed with silicone rubber caulking to prevent occasional leaks.

Potential problems exist in using commercially evacuated tubes for storage. The tubes are not completely evacuated and, thus, contain a significant amount of gas before the sample is added unless the tubes are reevacuated. If it is not possible to reevacuate the tubes, the initial internal pressure can be measured and a correction factor used in calculations. This initial dilution does not affect the $^{15}X_N$ calculations (see Table I for definition of terms) but does reduce the calculated d value. A second problem with the commercially evacuated tubes is that the N_2O content of the tubes is above that of normal air. When tubes are filled with 12 ml of normal atmospheric air, the tubes contain about 1000 ppb (v/v) N_2O rather than the about 310 ppb normally found in air. This is not a problem when measuring total N gas on the IRMS but must be corrected for when quantifying N_2O emissions from the soil. Corrections are made by collecting time zero gas samples from the field plots at each sampling time and analyzing them to determine the tube N_2O content of time zero air.

To illustrate the use of this ^{15}N technique to directly quantify N gases produced during nitrification–denitrification reactions in the soil, we will describe three case studies that include different agricultural systems: aerobic and anaerobic soils, a methods comparison, and studies where ^{15}N balance calculations were made.

2. Field Case Study 1

The details of this study are given in Mosier *et al.* (1986a,b). This field experiment consisted of three parts: N-gas flux determinations during one corn and one barley cropping season, N balance in the barley crop, and N gas flux in a fallow (uncropped) soil where the ^{15}N technique and the acetylene block method were directly compared.

Microplots were established inside a barley, a corn, and an adjacent fallow field by driving 15-cm-diameter × 45-cm-long plastic irrigation pipe sections into the soil. One corn plant or six barley plants were grown inside each cylinder. The top 10 cm of soil in the cylinders was removed and discarded. Soil collected prior to fertilization from the same site was amended with an equivalent of 200 kg N ha^{-1} as 99 atom% ^{15}N-$(NH_4)_2SO_4$, mixed thoroughly in a twin shell blender, and placed in the top 10 cm of the field cylinders. In the planted field, normal tillage and irrigation practices were performed during the growing season. Each individual treatment was replicated four times. In the fallow plots, eight microplots were instrumented for acetylene additions. Acetylene was injected into the soil in each cylinder through three separate nylon tubes (35 cm long by 4 mm ID) that had been previously drilled with 1-mm-ID holes 1 cm apart beginning 5 cm below the soil surface before inserting the tubes vertically into predrilled holes in the microplot soil. A gas chromatographic septum was fitted to the top of the tube to permit acetylene addition through a syringe needle. At the start of the fallow soil experiment, water was added to bring the top 20 cm of soil to about 33 kPa tension. Seventy cubic centimeters of acetone-free acetylene was added to each of the three nylon tubes per plot 1 hr before each gas sampling period. Two sets of four acetylene-treated plots were alternately used to minimize the effects of continuous exposure of microflora to acetylene.

Fluxes of N_2O and N_2 from soil were monitored by placing covers

Table IV
Data Used to Calculate Recovery of Applied ^{15}N-labeled Fertilizer, ^{15}N Balance, from the Field Plot Used in Field Case Study 2

Soil data

Soil depth (cm)	Bulk density (g cm^{-3})	Soil in layer (kg)	N content (mg kg^{-1})	N in layer (g)	Atom% ^{15}N fertilized	Atom% ^{15}N control
0–5	1.34	66.7	835	55.7	3.021	0.367
5–15	1.34	133	872	116	0.871	0.368
15–30	1.50	225	584	137	0.542	0.367

Plant data

Plant fraction	Yield[a] (g)	N content (mg Ng^{-1})	Total N (g)	Atom% ^{15}N fertilized	Atom% ^{15}N control
Straw	451	4.867	2.195	26.91	0.367
Grain	500	9.873	4.937	30.98	0.368

[a] Yield data are for N-fertilized plots.

(described earlier) over the microplots for 1 hr. Gas fluxes were measured two to four times each day following rain or irrigation (high flux rates) and three times every 7-day period at midday when there were small changes in field soil conditions (low flux rates). At the end of the growing season, whole plots were removed from the field and soils and plants analyzed for total N and ^{15}N (Porter and O'Deen, 1977).

The total N_2 + N_2O emissions for the cropping seasons were 4.5 and 1.5 kg N ha^{-1} from corn and barley plots, respectively. Of this total, about 1.5 and 0.7 kg N ha^{-1} was N_2 and the remainder N_2O. These small nitrification–denitrification-derived N emissions from the soil suggest that N losses by these mechanisms are not a major N loss mechanism from this irrigated, moderately well-drained soil. The ^{15}N balance data (see example in Tables IV and V) of the total ^{15}N in plot and surrounding soil and plants indicate that the total N-gas flux estimates were reasonably accurate. The recovery of added ^{15}N from the microplots averaged 96%. N-gas flux from the ^{15}N-fertilized fallow microplots was much larger than from the planted soils, thus providing a more descriptive example of the analytical technique. The fallow study also provides a comparison of the ^{15}N method with the acetylene block method for measuring N-gas production from denitrification. Figure 3 shows the total N-gas flux from fallow microplots following three irrigation events. N_2O and N_2 fluxes followed the same temporal

Table V
Calculating Recovery, ^{15}N Balance, of ^{15}N-labeled Fertilizer Applied in Crop and Soil at the End of a Growing Season

Using data from Table IV for soil and plant material as an example, ^{15}N recovery calculations are as follows.

A. Calculate amount of ^{15}N in (0–5 cm) soil from added fertilizer.
  1. Calculate amount of dry soil in 0–5 cm, plot area = 1 m^2, volume of soil = 50 liters, soil bulk density = 1.34 kg. Liter^{-1} g soil 0–5 cm = 1.34 kg liter^{-1}*50 liters = 67 kg.
  2. Measure total N content of the soil; 0.835 g N kg^{-1}.
  3. Total N in 0–5 cm = 67 kg*0.835 g N kg^{-1} = 55.9 g N.
  4. Measure atom% ^{15}N of soil total N; 3.021 atom% ^{15}N.
  5. Calculate amount of ^{15}N in soil: g ^{15}N = atom% ^{15}N/100*g N in soil = 0.03021*55.9 = 1.689.
  6. Calculate amount of N derived from fertilizer:
    a. Measure atom% ^{15}N of soil in plot where no ^{15}N fertilizer was applied (check plot); 0.367 atom% ^{15}N.
    b. Calculate amount of ^{15}N in check plot soil, 0–5 cm: g ^{15}N = 55.9 g N*0.00367 = 0.205 g.
  7. Calculate amount of ^{15}N in soil derived from fertilizer:
     ^{15}N from fertilizer = total ^{15}N − check plot ^{15}N = 1.689 g − 0.205 g = 1.484 g.

B. Calculate amount of ^{15}N in rice grain derived from fertilizer:
  1. Measure grain total N content; 9.873 mg N g^{-1}.
  2. Total N in grain over plot: 500 g*9.873 mg N g^{-1} = 4.937 g N.
  3. Total amount ^{15}N in grain = grain N*atom% ^{15}N of grain/100 = 4.937*0.3098 = 1.529 g N.
  4. Calculate ^{15}N derived from fertilizer:
    a. ^{15}N from check plot grain = total N*atom% ^{15}N/100 grain from check plot = 4.937*0.00368 = 0.018 g.
    b. ^{15}N derived from fertilizer = total ^{15}N − ^{15}N from check plot grain = 1.529 g − 0.018 g = 1.511 g N from fertilizer.

C. Calculate total ^{15}N derived from fertilizer in plot:
  Total ^{15}N = sum of fertilizer-derived ^{15}N from plant material and soil in the plot
  = ^{15}N from soil (0–5, 5–15, 15–30 cm) + ^{15}N from plant (straw + grain)
  = 1.484 + 0.614 + 0.276 + 0.582 + 1.510
  = 4.466 g ^{15}N.

D. Calculate % recovery of applied fertilizer in the plot:
  1. Amount of fertilizer applied, 10 g N m^{-2}.
  2. Amount of ^{15}N applied = g N*atom% ^{15}N/100
     = 10 g*0.99 = 9.9 g ^{15}N.
  3. % of fertilizer N recovered = 4.466 g/ 9.9 g*100
     = 45.11.

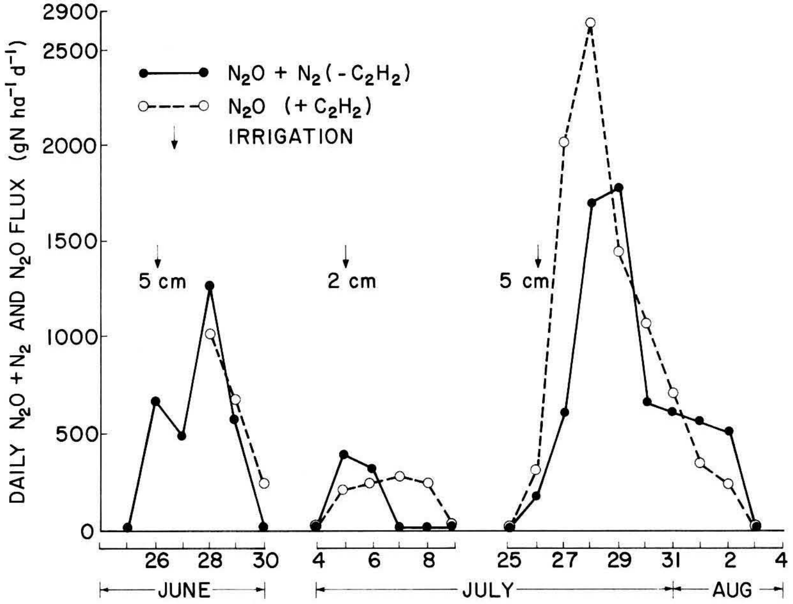

Figure 3 Total N-gas flux from C_2H_2-treated and untreated ^{15}N-fertilized field soil during three irrigation events. Gaseous $N_2 + N_2O$ from the untreated soil was measured by mass spectrometry, and N_2O from the C_2H_2-treated soil was measured by GC. [From Mosier, A. R., Guenzi, W. D., and Schweizer, E. E. (1986). Soil losses of dinitrogen and nitrous oxide from irrigated crops in northeastern Colorado. *Soil Sci. Soc. Am. J.* 50, 831–833].

patterns and N_2 emissions exceeded N_2O by an average of 10.3 times. Ratios of N_2/N_2O varied from 0 to 60 during the three periods of high N-gas flux. The proportion of N_2 was highest during intense emission periods and fell to zero or below the N_2 detection limit as the soil dried.

The total mean $N_2 + N_2O$ loss measured by IRMS was 13 and 25% less than that measured by the acetylene inhibition method for the first and second irrigation events, respectively. The amount of N lost during these two events was small compared to the amount of N lost following the third irrigation. About 28% more N was lost from the acetylene-treated plots during the third event. The relative difference in $N_2 + N_2O$ production using the direct IRMS analysis and the acetylene inhibition method is statistically significant ($p < 0.1$) and is similar to those shown in the lab case study 1 using soils from the same field site (Haider *et al.*, 1983a).

To further evaluate the merits of the ^{15}N technique, the gases collected from the acetylene-treated plots were also analyzed by IRMS during the third irrigation event so that the spatial variability aspect of the methods could be eliminated. The total $N_2 + N_2O$ evolved from the acetylene-treated plots during the 26 July to 2 August period averaged 8950 and 8740 g N ha^{-1} for the acetylene and ^{15}N methods, respectively, and the fluxes determined by the two methods were not significantly different ($p > 0.1$). There were small plot-to-plot differences between analytical methods in the cumulative gas flux determination because these data include the sum of all analytical errors accumulated from 14 different samplings taken during the 8-day period. Aulakh *et al.* (1991) further demonstrated the utility of these techniques for quantifying denitrification in the field.

3. Field Case Study 2

The second illustration is from a study conducted in a dry season rice field in Cuttack, India, where the soil was intermittently flooded (Mosier *et al.*, unpublished). This study shows how the ^{15}N method can be used in field sites far from the analytical laboratory and also that the direct gas flux measurements and total N balance at the end of the growing season does not always agree. The field plot was located at the Central Rice Research Institute. The soil is a Aeric Fluvaquept, which is only slowly permeable to vertical water movement. Inside a 5- × 20-m field plot, a 0.96-m^2 plot was isolated by driving a rectangular steel confinement 30 cm into the soil. This small plot was covered when urea (100 kg N ha^{-1}) was surface-applied to the entire field plot and 4-wk-old rice seedlings were hand-planted (10 cm apart with 20 cm row spacing). Inside the small plot, an equivalent amount of N (99 atom% ^{15}N-urea, 19.86 g) was uniformly surface-applied and rice seedlings planted into the soil slurry. The standing water was about 3 cm deep at planting, on 10 March 1987, Julian Day 69.

Measurements of N-gas flux (N_2 and N_2O) were begun during the afternoon, about 4 hr after urea was applied. The gases were sampled using rectangular chambers, 14 cm wide × 40 cm long and 12 cm high, which were made from plate glass glued together and the joints were sealed with silicone rubber caulking. Two holes were made in the top of each chamber to accommodate rubber septa through which a needle could be inserted to withdraw a gas sample. Four chambers were placed over the soil and inserted into the soil so that a 9-cm headspace depth existed. The serum stoppers were left out while the chambers were installed to prevent a pressure buildup. An initial gas sample was withdrawn by syringe from each chamber immediately after installation, the gas was injected into a freshly evacuated Vacutainer®, and the tube's rubber septum was immediately coated with silicone rubber sealant. The following sampling schedule was performed daily. Midafternoon, chambers were installed between the rice rows and gas samples removed after 0, 1, and 2 hr. Then, the chambers were removed from the plots. During early evening, chambers were installed in the plots, two between the rice row and two in the rice row enclosing three rice plants. Samples from these chambers were withdrawn after 0, 4, and 15 hr.

The resealed Vacutainers® were shipped to Ft. Collins, Colorado, where the collected gas was analyzed for N_2O by GC and for total N gas by IRMS. At plant maturity, the crop was harvested from the plot, separated into grain and straw, dried, and finely ground (100–200 mesh). The 0.96-m^2 plot was destructively sampled by excavating the entire plot in 0–5-, 5–15-, and 15–30-cm-depth intervals. The soils were air-dried, mixed thoroughly, and finely ground. Portions of the soil and plant material were shipped to Ft. Collins, where total N and ^{15}N content were determined by automated combustion-mass spectrometry (Marshall and Whiteway, 1985).

Cumulative N-gas flux for the cropping season totaled about 6 kg N ha^{-1} (Fig. 4). About 94% of this N was N_2 and 6% was N_2O. The ^{15}N balance (the total ^{15}N found in plant and soil from the ^{15}N-fertilized plot, example calculations for which are given in Tables IV and V) indicated that about 55% of the added N was not recovered in plant or soil. This indicates that if the fertilizer N was lost as a result of denitrification then the total gaseous N measured should have been about 10 times larger than the direct measurements indicated. The reason for this discrepancy is at this time unresolved and is the focus of some studies. Lindau *et al.* (1988) showed that significant quantities of N_2 produced from NO_3^- reduction are trapped in the soil. It is not known how long it takes for such entrapped gases to diffuse to the soil surface. Gas collections were made throughout the entire growing season even though no N emissions larger than 10 g N ha^{-1} day^{-1} were detected later than 30 days after transplanting. Other loss mechanisms such as NO_3^- leaching, lateral movement out of the plot, NH_3 volatilization, and NH_3 evolution from senescing rice plants were not measured. Ammonia volatilization is considered the likely loss mechanism.

4. Field Case Study 3

The third field case study was conducted in a rice field near Griffith, N.S.W., Australia. The purpose of this study was to determine the extent of urea N utilization when urea is applied in the floodwater to rice at the panicle initiation stage. At this stage of plant growth, N supply is very important to both grain yield and N content. This study (Mosier *et al.*, 1989) illustrates the use of ^{15}N to quantify N gas emissions from the floodwater, to quantify the amount of N_2 and N_2O dissolved in floodwater and soil pore water, and to quantify plant N uptake and residual fertilizer N in the soil and floodwater.

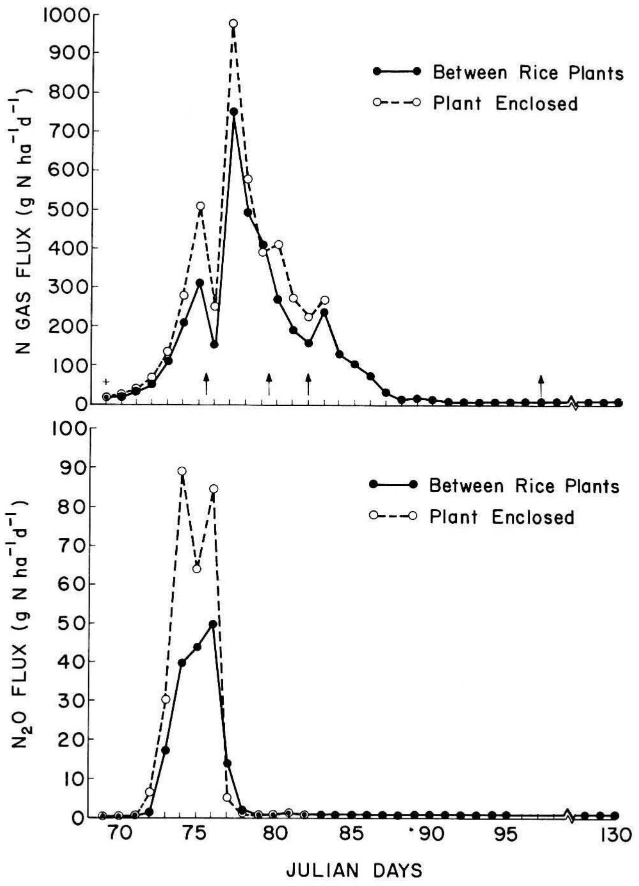

Figure 4 (A) Total N gas (N_2 + N_2O + N oxides) and (B) N_2O emissions from a ^{15}N-fertilized rice field.

On 23 February 1987, three microplots were established in a 10-ha rice bay by pushing stainless-steel cylinders (33 cm diameter × 60 cm high) 15 cm into the soil. The floodwater was maintained 20 cm deep. Four rice plants were confined within each cylinder. Cylinders were fertilized with 80 kg urea N ha^{-1} (79.2 atom% ^{15}N) by adding an aqueous urea solution to the floodwater confined in each cylinder. Gas flux measurements were made by periodically covering the microplots with a steel lid that was sufficiently large in diameter to rest flat against the upper edge of the plot enclosure (plants were folded over under the lid). A sampling port extending from the lid was sealed with a rubber serum stopper after the lid was installed each time. A steel flange, 15 cm long extended down from the lid to form a 30-cm-diameter cylinder. The lower 4 cm of the flange was below the water surface. This provided a sealed chamber of 7.775 liters volume from which 40-cm^3 gas samples were drawn by syringe and the gases were stored for later analyses in freshly evacuated Vacutainers® (Section IV.B.1). The collection covers were generally placed on the plots at 1400 hours and samples collected after 0, 1, and 2 hr. The covers were then removed and replaced at 1800 hours. Samples were collected after 0, 4, and 14 hr, after which the covers were removed.

Seven days later, the ^{15}N-labeled plots were destructively sampled. First, two 10-cm-diameter × 45-cm-long plastic tubes were driven into each plot (the samples included the standing water and 15 cm of soil, to permit analysis of the N_2 contained in the pore water and standing water). After removing and sealing both ends of these tubes, the standing water was siphoned off, the volume measured, and a 1-liter aliquot taken for analysis. The plants were pulled out of the soil and bagged, and then the top 15 cm of soil was removed and bagged. These samples were returned to the laboratory where the water, soil, and plant samples were analyzed for mineral N, total N, and ^{15}N. The small core standing water N_2 and $^{15}N_2$ content was extracted with He using a multiple equilibration technique (McAuliffe, 1971) and analyzed by GC and IRMS. The soil pore water was expelled from the soil taken in the 10-cm cores using a soil press with He gas at a maximum of 269 kPa. The pore water was then extracted with He and the He analyzed for N_2 and $^{15}N_2$.

Measurement of N gas emissions from the rice field floodwater were begun 22 hr after urea was added. The first sampling occurred after the cover had been in place 14 hr and indicated that N-gas emissions began quickly after urea addition. The mean daily N-gas flux, determined from overnight enclosure samples, was largest during the second day after urea addition, 560 g N ha^{-1} day^{-1} and decreased about fivefold by the seventh day. Cumulative gas flux was 2900, 1500, and 2700 for each of the three plots and averaged 2300 g N ha^{-1} day^{-1} for the 7-day period. Thus denitrification occurred rapidly after urea addition but did not continue at the highest rate for a long period.

We found that about 480 mg N_2-N/plot was dissolved in the standing water, but only about 0.04% of this N came from the applied urea. About 10 times less N_2 was dissolved in the soil pore water, with about 0.04% derived from the added fertilizer. The amount of N_2 dissolved in the floodwater was near the theoretical value for 20°C and 740 mm Hg pressure.

Total cumulative N_2O evolution averaged only 20.1 g N ha^{-1} during the 7-day collection period. N_2O concentration was greatest during the first sampling period and rapidly decreased to <2 g N ha^{-1} day^{-1} after 5 days. Unlike the total N flux that remained relatively constant regardless of the length of sampling period during each day, the N_2O chamber headspace concentrations did not increase linearly with time. For example, during 25 February, the plots were covered during two periods: from 1400 hours to 1600 hours and from 1800 hours to 0845 hours the next day. The N_2O flux during the first period were 11.6 and 6.8 g N ha^{-1} day^{-1} for 1 and 2 hr, respectively, while the flux rates were 10.2 and 7.4 for periods of 4.5 and 14.75 hr, respectively. This suggests that N_2O was being reduced to N_2 during the sampling period or that increasing headspace concentrations reduced outward diffusion of N_2O.

At the end of the 7-day sampling period, we found about 54% of the applied ^{15}N as ammonia (34% in floodwater and 20% in soil). Plant uptake of the added urea N was apparently rapid as an average of 21.1% of the applied N was found in the rice plants. A surprisingly large amount of the urea added (18.3%) was recovered in the organic N fraction of the soil. Although readily measured during the sampling period, the N-gas emissions represented only 3.9% of the ^{15}N applied. Total ^{15}N recovery on the plots averaged 97.3%.

V. CONCLUSIONS, PROBLEMS, AND FUTURE TOPICS

The examples presented in this chapter are principally from agricultural systems where fertilizers are normally used. There are two reasons for this emphasis. First, relatively few studies have been done using isotopes to measure nitrification or denitrification in natural ecosystems [although see Schimel (1986, 1987) for nitrification]. Second, the low rates of N-gas flux from many natural ecosystems (Bowden, 1986) make isotope techniques more difficult to apply. In principal, there are no major obstacles to using ^{15}N measures of denitrification in natural ecosystem types where rates of emission are relatively high (tropical ecosystems), and tracer-level (rather than plant fertilizer levels) additions of ^{15}N would provide sufficient analytical sensitivity. Indeed, isotope studies of denitrification and N_2O emission from natural ecosystems are badly needed to improve understanding of global trace gas emissions.

Problems exist with isotopic techniques. In natural ecosystems and agricultural ecosystems where emission rates are relatively low, directly measured gas emission and isotopic N balance agree well. In systems where N losses are high, the lost N cannot be accounted for by direct gas flux measurements. Because only a few studies have been conducted, we have yet to learn if this is due to methodological problems, or if loss via vectors not simultaneously measured occurred. Problems could also result from interference with gas movement in soils by the chamber employed, nonuniform mixing of isotope in soils, or other factors. The effects of mineralization, immobilization, and turnover of N and ^{15}N enrichment of soil NO_3^- and NH_4^+ and uniformity of isotopic distribution will change over time. We note that very few studies have been conducted comparing mass balance and gas flux measurements. Room for considerable development of field and modeling techniques exists.

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8

Mineralization and Assimilation in Soil–Plant Systems

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I. INTRODUCTION

In most terrestrial ecosystems, whether natural or agricultural, soil organic matter constitutes by far the largest pool of combined nitrogen, excluding that in rocks (Porter, 1975). The proportion of this nitrogen undergoing some form of transformation, such as mineralization, in a year may be only a few percent but, because the pool is so large, the quantity thus transformed is large, often of the same order of magnitude as plant uptake or nitrogen fertilizer application. Using ^{15}N as a tracer enables transformations of added fertilizer N to be measured against the enormous background of soil N. However, soil is a biological system, not an inert medium, so any tracer nitrogen added immediately becomes enmeshed in the biological and chemical transformations that are occurring. The tracer can become a valuable tool for probing soil processes and yet the very transformations of interest greatly complicate the interpretation of results from ^{15}N experiments—This will be considered later.

II. PRELIMINARY CONSIDERATIONS

A. Purpose of Experiment

Before deciding to use ^{15}N, it is worth considering nonisotopic techniques because the use of ^{15}N does not automatically improve the quality or value

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of the results obtained, especially if it is *only* plant uptake of fertilizer N that is to be measured. Two nonisotopic techniques are available for measuring uptake of fertilizer N by plants:

a. The control plot method. The total N uptake of a crop given no N fertilizer is subtracted from that of a crop that has received N fertilizer. The difference is taken as the amount of N derived from fertilizer.
b. The regression method. Several different rates of fertilizer N are applied to the crop, and the gradient of the regression of crop N uptake on fertilizer N applied is taken as the average fraction of fertilizer N recovered.

Both methods involve assumptions and have limitations. The obvious disadvantage of the first method is that considerable weight is placed on the N uptake of a crop receiving no N fertilizer. It is assumed that the uptake of N from soil, and any other nonfertilizer sources, is the same in the unfertilized and fertilized crops. In the regression method, data from the control treatment is given no more weight than data from any of the N applications.

The most serious limitation of nonisotopic experiments on plant uptake of fertilizer N is that they yield no information on the fate of the fertilizer N *not* recovered in crop, unless independent measurements of loss processes are made. The use of ^{15}N can provide some of this information but *only* if the quantity retained in soil is also measured. The use of ^{15}N avoids the assumptions inherent in nonisotopic methods but it does introduce another set of assumptions. Hauck and Bremner (1976) list 12 assumptions that are often made, even though some of them will be incorrect in a significant number of situations. In particular, the decline in ^{15}N abundance in the soil inorganic pool during an experiment due to mineralization of (unlabeled) soil N, and the removal of ^{15}N by processes other than plant uptake, can cause problems in interpretation. The erroneous conclusions that result from making incorrect assumptions are particularly serious if only crop uptake of ^{15}N is measured; this is discussed more fully in Section VI.

If ^{15}N will be used, it is important to set clear aims for the study because these will determine the most appropriate experimental approach. A possible classification of experimental aims is given below.

1. Mechanistic Studies

^{15}N can be used to see whether or not N is transferred or transformed from one place, chemical form, or metabolic pool to another within a soil–plant system. In such studies, it may not be necessary to obtain a complete or quantitative account of the distribution of ^{15}N but, rather, a yes or no answer as to whether or not the tracer can be found in a particular place or form. Examples include studies of metabolic pathways within plants, determination of whether or not atmospheric N_2 is biologically fixed in a given situation, and the mechanisms and intermediates of nitrification or denitrification in soil.

2. Rate Measurements

The measurement of the rate or extent of a given process (e.g., mineralization, nitrification, plant uptake) requires quantitative measurement of ^{15}N in one or more places, chemical forms, or pools. Many agricultural experiments fall within this category, and the aim is often to see how a process is altered by particular soil, plant, management, or climatic variables.

3. ^{15}N Balance

In such studies, a complete quantitative account is required of the ^{15}N in as many parts of a soil–plant system as possible. Measurements of the rate or extent of several different processes is obtained simultaneously and the amount of ^{15}N *not* accounted for is calculated. This category includes agricultural and environmental studies concerned with losses of N and the fate of fertilizer.

B. Scale of Experiments

Laboratory incubations of soil, and growth chamber or glasshouse experiments with plants in pots, are well suited to detailed mechanistic studies of nitrogen transformations. Many factors can be held constant to increase the possibility of detecting processes of interest with minimal interference from others. In these experiments, the researcher is asking questions such as "what processes *can* occur?" and "what factors control them?" However, for many purposes it is necessary to measure processes under more realistic conditions—to ask questions about the processes that actually *do* occur in a given environment, their rates, relative importance, and the factors influencing them. This generally requires experiments to be conducted under field conditions, but field experiments present the greatest number of obstacles to the achievement of a clear result for the following reasons:

a. Many variables are outside the researcher's control, so it can be difficult to assess the importance of individual factors on the processes being studied.
b. ^{15}N-labeled material must be applied evenly and accurately to a substantial area.
c. Representative samples of plant material and soil are required despite possible edge effects and spatial variability both within and between field plots.

The ideal is to apply ^{15}N to an area that is large in comparison to the spacing of plants and to sample only the center of this area: This is perfectly feasible for many crops such as grass, cereals, and vegetables. With large or widely spaced plants such as sugar cane or trees, it may only be feasible to treat individual plants with ^{15}N, in which case the plant can be surrounded by a plastic or metal barrier (e.g., Melin *et al.*, 1983; Saffigna and Wood, 1986). A smaller-diameter (10–30 cm) plastic or metal tube that can be removed in its entirety for analysis is sometimes inserted into soil to provide an enclosed area for a ^{15}N study (e.g., Riga *et al.*, 1988). While this can be useful, possible artefacts should be considered such as interference with root growth or water movement. Parkin and Codling (1988) noted that chemical reduction of nitrate occurred due to iron and zinc from a galvanized steel barrier.

Lysimeters of various types offer another compromise between the well-controlled, but artificial, conditions of a laboratory or growth chamber experiment and the more natural, but chaotic, conditions of the field. A particular advantage of lysimeters is that drainage water can be collected and analyzed. This facilitates a more complete account of nitrogen in ^{15}N balance studies than is possible with field plots; lysimeters can also be designed so that gaseous losses can be measured; isotopic methods for estimating gaseous losses are described in Chapter 7, Section III. A limitation of lysimeters is the risk that greater cracking of soil will occur than in the field, especially shrinkage of soil away from the lysimeter walls. This can alter the flow of water and hence the leaching of nitrate (Powlson, 1988). Water can also be held back in a lysimeter because of the absence of capillary contact with deeper soil unless tension is applied (van Bavel, 1961). As is common in science, the researcher has to evaluate the relative importance of the advantages and disadvantages of the various available experimental procedures for his particular study. Saffigna (1988) discusses the merits and limitations of the various options for ^{15}N experiments in greater detail.

III. PROCEDURES FOR FIELD EXPERIMENTS WITH ^{15}N

A. ^{15}N Enrichment of Starting Material

It is necessary to estimate the likely degree of dilution of tracer when deciding on the enrichment to use. With many common field crops, fertilizer-derived N in the plant is diluted two- to fourfold with unlabeled N derived from soil. In studies with organic sources of ^{15}N, in which the release of inorganic N may be slow, dilution could be much greater. This is also the case with trees, which will have a large initial content of unlabeled N. However, the greatest dilution occurs in soil, and this will often determine the enrichment necessary.

Consider a labeled fertilizer application of 100 kg N ha^{-1} to two soils, one containing 2000 kg N ha^{-1} in the surface layer and the other 10,000 kg N ha^{-1}, corresponding to approximately 0.1 and 0.5% total N. If the fertilizer is applied at 5 atom% excess and 20% is retained in soil organic forms at the end of the experiment, the ^{15}N enrichment in the two soils will be approximately 0.05 and 0.01 atom% excess, respectively. The former value is a reasonable enrichment to measure but the latter is so low that accuracy may be poor and it would be preferable to apply label at a higher enrichment.

B. Application of Labeled Material

For quantitative work, an accurately known amount of labeled material must be applied to a plot. In addition, distribution within the plot must be uniform if, as is usually the case, only the central area is to be sampled. Accurate and even application is equally important for agricultural experiments with labeled fertilizer and for experiments on natural ecosystems in which a small quantity of heavily labeled N is applied to introduce tracer to a soil pool. Extremely uniform application is less critical in pot, lysimeter, or confined microplot experiments in which all of the plant material or soil from the container is taken for analysis. In field experiments on topics such as fertilizer placement, application is inevitably nonuniform and special care must be taken to obtain representative samples (Moraghan *et al.*, 1984a,b).

1. Application of Solid

In agricultural practice, fertilizers such as urea and ammonium nitrate are usually applied as solids so there is good reason for applying labeled fertilizers in the same way (Smith *et al.*, 1984), but achieving even application within a plot can be difficult. The labeled solid can be mixed with an inert carrier such as sand so that a larger quantity of material is applied and its distribution can be seen more easily during spreading. The plot can be subdivided into areas more convenient for hand application, each of which receives a preweighed quantity. Labeled fertilizer materials in forms such as granules, with the same physical characteristics as commercial fertilizers, can be obtained from the Tennessee Valley Authority and should be of value in experiments comparing different fertilizer formulations or placement techniques.

2. Application of Solution

Application of solution is the most common method because it is easier to achieve even application of a solution than a solid. Labeled solution can be applied with a watering can but, unless extreme care is taken, the quantity of solution applied per unit area may differ between the edges and the center. This can seriously affect the results because the *actual* application rate in the central area, which is later sampled, is different from the average rate for the plot as a whole, which is used to calculate percentage recovery of the applied ^{15}N. A more satisfactory method is to subdivide the plot and apply an equal volume to each area with a pipette or automatically refilling dispenser (Saffigna, 1988). A plot can be subdivided into areas of about 30 × 30 cm using a wooden frame supporting a lattice of wires or strings; about 10–30 ml for each area is a convenient volume. If the number or size of plots is too great for this method to be practical, a hand-operated applicator such as that described by Woodcock *et al.* (1982) is useful. This comprises a peristaltic pump that directs solution from a reservoir (5 or 10 liters) to a dribble bar consisting of 20 jets at 5-cm spacing. A hand-operated rack and pinion system, with appropriate gearing, operates the pump and simultaneously moves the dribble bar along a frame. Solution is applied to a 1 × 2-m area but larger plots can be treated by accurately repositioning the spreader. Although this device applies ^{15}N-labeled solution in bands 5 cm apart rather than completely evenly, this was considered suitable for experiments with crops in which sampling would be several weeks later; after a period of crop growth and N uptake, the distribution of residual ^{15}N in soil reflects plant distribution rather than the original banding. An advantage of the apparatus described by Woodcock *et al.* (1982) is that even and accurate application does not depend on a constant speed of operation because the pumping rate and the speed of movement of the dribble bar are linked. Other, simpler, devices can be constructed that deliver solution at a constant flow rate, but uniform application to a plot depends on the device being moved across the plot at a constant velocity.

If the aim is to mimic the normal application of fertilizer, which would be as granules, the volume of solution must be kept to a minimum so that the soil is not made abnormally wet. If this is not done, there is a risk of denitrification or lateral flow of water. The apparatus of Woodcock *et al.* (1982) delivers about 250 ml m^{-2} from a single traverse, equivalent to 0.25 mm of rainfall. This is usually followed by up to 500 ml m^{-2} of water from a watering can to wash any labeled solution off plant foliage. With an application of 144 kg N ha^{-1} to wheat at growth stage (G.S.) 22 (about 15 cm tall) only 1.5% of the ^{15}N applied remained on the plants (Powlson *et al.*, 1986).

Spraying of solutions can also be used to obtain even application to a plot. By using a low-volume sprayer, many passes can be made so that application will be more uniform than with a watering can. Recous *et al.* (1988a) used a spray technique in which plastic barriers were placed at the edges of the plot to prevent drift. Eight liters of labeled solution were sprayed onto a 3.5 × 3.5-m plot followed by 2 liters of water. This technique proved satisfactory except that there was evidence of foliar uptake by wheat when labeled N was applied to wheat at the beginning of stem extension (Recous *et al.*, 1988b). Thus, there is a risk that the fate of labeled N sprayed onto a crop will not be representative of granular fertilizer applied to the soil.

Spraying can, of course, be used to investigate the uptake of N from foliar applications. Powlson *et al.* (1987, 1989) describe two techniques for this. In the first, a precision sprayer was used to deliver ^{15}N-labeled urea solution to wheat at around the time of anthesis (G.S. 65); the application volume was about 230 ml m^{-2} to plots of 10–16 m^2. This volume was greater than is usual in agricultural practice because, with the sprayer used, it was found impossible to achieve even coverage with a smaller volume—Other sprayers may be more satisfactory. Before using a sprayer, it should be checked carefully for uniformity of application; for example, the volume of solution delivered by each jet along the spray boom can vary considerably. Another disadvantage of this technique is that even application depends on the spray operator walking at a constant speed. In the second technique, a hand sprayer was used to apply labeled solution (at 100 ml m^{-2}) to an area of 2 m^2 within a larger plot, the remainder of which received unlabeled foliar-applied N. The advantages and disadvantages of this technique are discussed in Section III.C.1. In addition to fertilizer experiments, sprays may be a useful means of introducing a single pulse of ^{15}N into a plant to study translocation within the plant or exudation into soil. Janzen and Bruinsma (1989) describe a technique for exposing plant tops to air containing ^{15}N-labeled NH$_3$ gas so that translocation of N from tops to roots, and exudation into soil, can be measured. Initial experiments indicate that amounts of N equivalent to about 20–30% of the N in plant tops was liberated into the soil by young wheat plants (Janzen and Bruinsma, 1989; Janzen, 1990).

Techniques have also been developed to label fertilizer N that is applied in specialized ways including injection of anhydrous ammonia (Sanchez and Blackmer, 1987) and "fertigation," in which ^{15}N-labeled urea is dissolved in the irrigation water of a drip irrigation system for sugar cane in Mauritius (Ng Kee Kwong, personal communication). In Finland, M. Esala (personal communication) simulates band application of fertilizer by making a trench (5–10 cm deep) between rows of a cereal and using a repeating dispenser to deliver a predetermined volume to a given length of row.

3. Application of Labeled Plant Material

^{15}N-labeled plant material can be used to study the release of N as it decomposes in soil and its subsequent transformations. The need for even application within a plot is as great as in experiments with solutions but is harder to achieve. It may be necessary to chop the plant material more finely than would be the case in normal practice in order to achieve uniformity; if so, it may also be necessary to conduct laboratory or glasshouse studies to see whether this alters the rate of decomposition.

Plant material is usually incorporated into soil to some extent by digging. If, however, it is desired to simulate a zero tillage situation in which it remains on the surface, it may be necessary to cover the plot with netting to prevent loss (White *et al.*, 1986). Plant material can also be buried in mesh bags; this is useful if the aim is to study chemical changes in the plant material, its microbial colonization, the involvement of soil fauna in decomposition or the release of soluble N into soil.

C. Size of Plot

1. Plots with Discard Area

With unconfined field plots, some lateral movement of ^{15}N by diffusion, mass flow, or translocation after plant uptake is virtually inevitable. Even in the absence of such movement, plants near the edge of the ^{15}N-treated area will derive some of their N from outside the plot, thus decreasing their ^{15}N enrichment. Because of this, it is necessary to exclude plants from near the edge of the plot from the sample taken for analysis. The size of plot and discard necessary for representative and reproducible results is largely determined by the size and spacing of the plants being grown but may also be affected by soil properties and weather (particularly the quantity and intensity of rainfall) because these will influence the lateral movement of ^{15}N. Plots as small as 1 × 1 m have been used for grass and plots of about 2 × 2 m, with a central sampled area of about 1 × 1 m, have commonly been used for cereals. For maize, which is more widely spaced, plots of 3.56 × 3.56, 4 × 4, and 3 × 2 m have been used (Olson, 1980b; Khanif *et al.*, 1984; Chabrol *et al.*, 1988). With a 2 × 2-m plot comprising 12 rows of wheat on a silty clay loam soil, Powlson *et al.* (1986) showed that a sampling area of 1.07 × 1.07 m (six rows) was adequate for avoiding edge effects; grain from two rows outside the central area had the same ^{15}N enrichment as that from within it (Fig. 1). Olson (1980a) applied ^{15}N to a 3.56 × 3.56 m plot comprising five rows of maize at 71-cm spacing between rows. Only plants from the outermost rows had a lower ^{15}N enrichment than those from the central row so one guard row appeared to

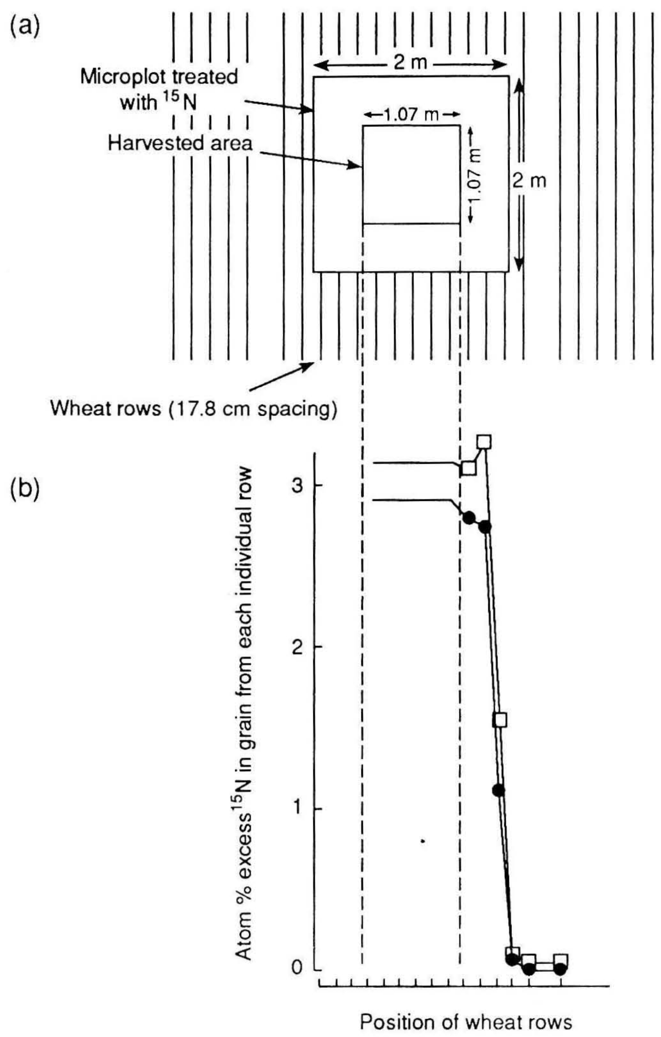

Figure 1 Boundary effects in a ^{15}N microplot sown to winter wheat. (a) Position of microplot and harvest area in relation to wheat rows. (b) Edge effects measured at harvest in the year of application. □—□, $K^{15}NO_3$ applied, ●—● $(^{15}NH_4)_2SO_4$ applied. [From Powlson, D. S., Pruden, G., Johnston, A. E., and Jenkinson, D. S. (1986). The nitrogen cycle in the Broadbalk Wheat Experiment: Recovery and losses of ^{15}N-labelled fertilizer applied in spring and inputs of nitrogen from the atmosphere. *J. Agric. Sci., Cambridge* 107, 591–609.]

be sufficient. Sanchez *et al.* (1987) reported a detailed study of plot-size requirements for maize and concluded that a 2 × 2-m plot is likely to be adequate for maize at a row spacing of 76 cm under most conditions. They suggest sampling some plants from inside and outside the labeled area. On the assumption that uptake of ^{15}N by plants at a given distance outside the boundary of the labeled plot is equal to uptake of unlabeled N by plants at the same distance inside the boundary, the adequacy of the plot size and sampling area can be tested and, if necessary, corrections made. Measuring the isotopic composition of plants outside the labeled plot provides greater sensitivity than equivalent measurements on plants inside the plot.

If a plot is used to follow the fate of labeled N over more than one growing season, cultivation will cause a significant spread of labeled soil. The discard boundary that is adequate for a single-year experiment may be too narrow to protect the central area from this dispersion over several years. Using 2 × 2-m wheat plots, with a 1 × 1-m central sampling area, Hart *et al.* (1992) estimated that the measured enrichment of plants within the central area was about 20% too low 5 yr after the original labeled application. They used a simple graphical technique, based on the enrichment of plants taken from outside the plot, to make a correction.

2. Plots without Discard Area

It is not uncommon for 75% of the labeled area to be discarded in the normal plot design in which only the central area is sampled. While this is acceptable for experiments with small plants such as arable crops, vegetables, or grass, it can make the cost of ^{15}N prohibitive for large or widely spaced plants. If it can be demonstrated that movement of ^{15}N between rows is small, it is possible to dispense with discard areas or guard rows. This has been shown for sugarcane in Mauritius, where the row spacing is 1.5 m and N fertilizer is banded along the rows. Two years after ^{15}N application, <30% of the applied N had moved >30 cm laterally from the fertilizer band (Ng Kee Kwong and Deville, 1987).

Powlson *et al.* (1989) described an approach termed the "negative discard method". This proved useful in experiments in which recovery of foliar-applied ^{15}N-labeled fertilizer by winter wheat was measured, but it could also be used in studies with soil-applied fertilizer if it is only the recovery in *crop* that is required. In this approach, labeled fertilizer is applied to a small area within a larger plot while unlabeled fertilizer, at the same rate, is applied to the surrounding area. The crop is sampled from an area that is larger than the labeled area and judged to be sufficiently large to include any labeled N that could have moved outside the original application zone. The application and sampling areas used by Powlson *et al.* (1989) were 2 and 4.5 m^2, respectively, but could be adjusted for other situations. The ^{15}N enrichment is measured in a subsample from the whole of the sampled area containing a mixture of labeled and unlabeled material. The practical benefits of this approach are as follows:

a. ^{15}N can be applied to a fairly small area without the need for a discard area, thus decreasing the cost.
b. Very uniform application of ^{15}N and a sharp boundary between labeled and unlabeled areas is less critical than for a normal plot with a discard.
c. Sampling may be simplified because of the larger area. For example, it was possible to sample wheat using a combine harvester designed for small plots (cutter width 1.5 m) instead of the more labor-intensive hand harvesting followed by threshing.

However, the technique has important limitations:

a. Very thorough mixing of the entire sample is necessary before subsampling because the sample contains both labeled and unlabeled material.
b. The analytical procedure used must be capable of measuring a fairly low ^{15}N enrichment with good precision because the labeled material is diluted by unlabeled.
c. It is not well suited to experiments in which the recovery of foliar-applied N in soil is required.

D. Sampling and Sample Handling

1. Plant Material

Avoiding inadvertent loss of plant material is particularly important in ^{15}N balance studies because the loss of ^{15}N would be misinterpreted as a loss from the crop–soil system in the field. Losses to be avoided include material that falls from plants during the growing season (e.g., dead leaves, shoots) and shedding of dry or brittle material during or after the sampling of mature crops (e.g., seeds, grains, pods, leaves). Further losses can easily occur during postharvest operations such as chopping or threshing. Specially designed, or modified, small-scale apparati may be required because those designed for nontracer agronomic experiments are often inadequate. For example, loss of chaff can easily occur when cereal samples are threshed. A second problem with these operations is the risk of cross-contamination; pieces of plant material can become temporarily trapped in an irregularly shaped interior and released into a later sample.

2. Soil

A sampling strategy must be adopted that is appropriate for the likely distribution of ^{15}N in soil. In general, the larger the quantity of soil that can be removed, mixed, and subsampled, the more likely the final analyzed sample will be representative of the plot. This can be achieved by taking either a large number of small soil samples from a plot or a smaller number of large ones from which a subsample is taken after mixing; excess soil can then be returned to the plot. The more uneven the distribution of ^{15}N in soil, the harder it is to obtain a representative sample; uneven distribution is most likely to occur where large or widely spaced plants are grown or where fertilizer was applied in bands or at distinct points. The presence of cracks or large stones increases the possibility of heterogeneity of ^{15}N distribution, as do tree roots in forest studies. To calculate the amount of labeled N present, it is essential to obtain an accurate value for soil weight on an area basis or of bulk density after allowing for the volume occupied by stones.

Soil near the surface (e.g., the cultivated layer of an agricultural soil, the zone of intense rooting) usually has a much higher ^{15}N enrichment than deeper soil. Consequently, any contamination of subsoil with topsoil greatly affects the results. One way of minimizing contamination is to use augers of decreasing diameter to take deeper samples. In the sampling technique described by Powlson *et al.* (1986), in which a petrol-driven posthole auger is used to obtain a large sample of surface soil from a 30-cm-diameter hole, the sides of this hole are lined before deeper drilling (at 15 cm diameter) commences.

If correct deductions on the loss of N are to be made from ^{15}N balance experiments, it is necessary to establish the distribution of labeled N with depth for a given soil–plant–environment combination. An appropriate sampling depth can then be selected. The background ^{15}N enrichment of N in soil organic matter is often >0.3663, the value normally taken as the natural abundance in atmospheric nitrogen. It is important to measure the exact background level for each soil depth because the measured enrichment in soil from a labeled plot, particularly of deeper soil, is likely to be only slightly above background.

Buresh *et al* (1982) advocate taking a zero time sample immediately after application of ^{15}N-labeled material to test the whole sampling, sample preparation, and analytical procedure for quantitative recovery. Although this is a useful precaution, it should be recognized that the distribution and chemical form of ^{15}N at this time will be quite different from that at the usual sampling time.

3. Sub-sampling and Grinding

The portion of soil or plant material that is ground and analyzed will be a very small proportion of that collected from a field plot, so care must be taken in every subsampling operation to ensure that the final sample is representative. It is advisable to use a "quartering" technique in which a sample is spread out on a flat clean surface and segments removed; sample splitters can also be used. These techniques overcome the problem of the subsample being biased toward particles of a particular size or shape; a major risk if a subsample is removed from a bag or bottle.

Fine grinding is essential for the small samples (typically 10–20 mg) used for Automated Nitrogen and Carbon Analyser-Mass Spectrometer (ANCA-MS) systems. The grinding method must also minimize the possibility of cross-contamination between samples. A disc-mill (Pruden *et al.*, 1985b) or ball-mill is much easier to clean between samples than a hammer- or knife-action mill and will usually achieve finer grinding. For grinding plant material in a disc mill, it is sometimes necessary to redry for a short time immediately before grinding. For samples that cannot be dried by heating, freezing in liquid nitrogen (C. Quarmby, personal communication) or freeze-drying can be used. R. D. Hauck (personal communication) uses a grinding technique in which samples are placed in screw-capped glass storage jars with lengths of steel rod of varying diameters. They are placed on a roller mill for several hours and extremely fine grinding is achieved.

IV. ANALYTICAL METHODS FOR DETERMINING TOTAL N AND ^{15}N ENRICHMENT IN PLANT AND SOIL

Methods are described in detail in Chapter 2, Sections III.B and IV; some comments of specific relevance to quantitative studies on mineralization and assimilation are given here. For such studies, the accurate measurements of the total quantity of N in soil, plant, or specific pools within the soil–plant system are just as important as measurements of ^{15}N enrichment. The analytical methods chosen must give a complete quantitative recovery of all forms of N in the sample. For example, if the Kjeldahl digestion method is used, a version that includes nitrate should be chosen; Pruden *et al.* (1985a,b) give two such methods. Particular care must be taken to avoid cross-contamination between samples of different ^{15}N enrichment. This can occur when Kjeldahl digests or soil extracts are steam-distilled (Pruden *et al.*, 1985b), when the acidified distillate is dried down prior to treatment with LiOBr in the inlet system of a traditional mass spectrometer (Lober *et al.*, 1987; Kelley, 1989) or between samples in an ANCA-MS system. If an ANCA-MS system is used for total N determination, in addition to ^{15}N enrichment, samples must be weighed very carefully using a five- or six-decimal place balance.

V. CALCULATION OF RESULTS

The ^{15}N enrichment of nitrogen in a sample from a tracer experiment represents a mixture of labeled N from the tracer, applied at a known enrichment, and unlabeled N containing ^{15}N at the background value characteristic of the material and environment. The amount of N derived from the tracer is calculated from the following equation given by Hauck and Bremner (1976) but using different notation:

$$F = \frac{T(A_s - A_B)}{A_F} \tag{1}$$

where F is weight of N derived from labeled fertilizer in crop or soil sample, T is total weight of N in sample [T and F are expressed in the same units (e.g., $\mu g\ N\ g^{-1}$ sample, mg N per pot, kg N ha^{-1}], A_S is atom% excess ^{15}N in labeled sample of crop or soil, A_B is atom% excess ^{15}N in control sample of crop or soil that did not receive labeled fertilizer (i.e., the background enrichment), and A_F is atom% excess ^{15}N in labeled fertilizer as added. Atom% excess is defined as the atom% ^{15}N in a material minus 0.3663.

It may be necessary to modify Eq. (1) to

$$F = \frac{T(A_S - A_B)}{A_F}\left(\frac{M_1}{M_2}\right)$$

This arises if, as is often the case when the Kjeldahl–Rittenberg method is used for analysis, sample total N content is calculated before its ^{15}N enrichment is known. If so, an erroneous value (usually 14) for the average atomic mass of N in the sample will have been used in calculating total N. The factor (M_1/M_2) corrects for this, where M_1 is the true average atomic mass of N in the sample and M_2 is the erroneous value used.

If the Kjeldahl–Rittenberg method is used, it will often be necessary to introduce another correction when using Eq. (1) to take account of the small amount of extraneous N that will inevitably contaminate the sample, mainly because of ammonia and amines dissolved in the sulfuric acid. This N is likely to be close to natural abundance and, thus, will decrease the ^{15}N enrichment of a labeled sample. The measured enrichment (A_M) will result from a mixture of N from the sample (quantity S, true enrichment A_S atom%) and from contaminant (quantity C, assumed to be at natural abundance, 0.3663 atom%) and is given by

$$A_M = \frac{S \times A_S + C \times 0.3663}{S + C} \tag{2}$$

S and C are expressed on the same basis (e.g., amount per Kjeldahl tube). The value of C, the quantity of contaminant N, can be obtained by measuring the decrease in enrichment of a standard solution of labeled $(NH_4)_2SO_4$ when it is measured after Kjeldahl digestion compared to the value obtained directly.

Let A_1 be ^{15}N atom% measured in the N_2 gas released by the reaction of LiOBr with a portion of labeled $(NH_4)_2SO_4$ solution that has been dried without prior digestion or distillation, A_2 be ^{15}N atom% measured after digestion and steam distillation of a portion of the same solution, Q be quantity of N taken for digestion. The measured enrichment of the standard solution after digestion (A_2) is given by

$$A_2 = \frac{Q \times A_1 + C \times 0.3663}{Q + C} \tag{3}$$

if it is assumed that contamination during reaction with LiOBr is negligible. The quantity of contaminant N per Kjeldahl tube, C, can be calculated and used in calculating the true enrichment of an unknown sample (A_S) using Eq. (1) or (2).

In the ANCA-MS method, such a correction is not needed if a standard of similar enrichment to the samples is used because both will be diluted similarly. If an unlabeled standard is used, a correction can be made using Eq. (2), but it is probably only significant for very small samples (see Section VII.C).

It is sometimes necessary to measure the isotopic composition of samples that contain very little total N (e.g., soil extracts, leachates or samples of soil solution containing inorganic N or soil extracts for biomass N determination). In these situations, contaminant N can be a larger proportion of sample N than in a regular Kjeldahl digest of plant or soil so the correction is relatively more important. If the quantity of N is too small for accurate measurement of ^{15}N on the mass spectrometer, the sample can be spiked with an accurately known quantity of unlabeled N, added as ammonium sulfate. In this case, the measured enrichment, A_M, refers to a three-component mixture and is given by the expression

$$A_M = \frac{S \times A_S + C \times 0.3663 + B \times A_B}{S + C + B} \tag{4}$$

where B is quantity of unlabeled N added as spike, expressed on the same basis as S and C; and A_B is atom% ^{15}N measured in the unlabeled spike, which usually differs slightly from 0.3663. The need for spiking is avoided if the dried residue from steam distillation is analyzed for ^{15}N enrichment using an ANCA-MS system.

VI. INTERPRETATION OF RESULTS FROM EXPERIMENTS WITH ^{15}N—THE ADDED NITROGEN INTERACTION

When labeled inorganic N is added to the pool of unlabeled inorganic N in soil, it is subject to various processes in addition to uptake by plant roots and translocation to tops. These include

a. immobilization into the soil microbial biomass or other fractions of soil organic matter,
b. Uptake and retention in roots,
c. leaching,
d. gaseous loss (e.g., ammonia volatilization, denitrification, loss of N oxides during nitrification), and
e. fixation of ammonium on clays.

Some immobilization will almost always occur (see references in Jenkinson *et al.*, 1985), as will retention in plant roots if plants are present. Whether or not the other processes occur in a given situation will depend on soil properties and environmental conditions.

Consider the simplified situation of a soil containing an amount P of unlabeled inorganic N to which an amount F of labeled inorganic fertilizer N is added. Assume that some immobilization occurs before plant uptake and removes an amount i of N from the inorganic pool. Furthermore, assume that the amount immobilized is the same whether or not fertilizer N is added. In the absence of plants, this is reasonable because immobilization is generally determined by the amount of decomposable carbon already present in the soil rather than by the amount of inorganic N present. However, in the presence of plants, there may be some additional immobilization where fertilizer N is applied. For example, rhizodeposition of carbon may be greater if the root system is larger in the presence of fertilizer. For the purposes of this example, mineralization of N (from both unlabeled soil organic matter and remineralization of labeled N) are ignored.

Where no labeled N is applied, the amount of unlabeled inorganic N still present after immobilization, and available for plant uptake, is $P - i$ (see Fig. 2a).

Where the quantity F of labeled fertilizer N has been added, the amount of inorganic N present after immobilization is $P + F - i$ (see Fig. 2b).

If it is assumed that labeled and unlabeled inorganic N are completely mixed, and indistinguishable to the immobilization process (or to plant uptake), the amounts of labeled and unlabeled N immobilized will be in proportion to the amounts of each present. Thus, the quantity of unlabeled

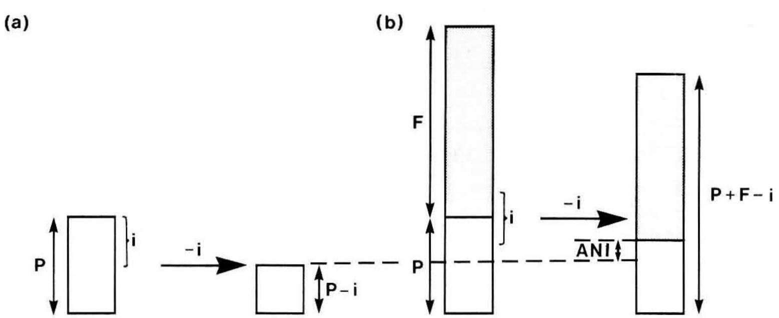

Figure 2 How pool substitution gives rise to an added nitrogen interaction (ANI). (a) Removal of a quantity i of inorganic N from unamended soil containing a quantity P of inorganic N. (b) Removal of an equal quantity i from soil to which an amount F of fertilizer inorganic N has been added.

inorganic N immobilized will be

$$i\left(\frac{P}{P + F}\right).$$

and the amount of labeled inorganic N immobilized will be

$$i\left(\frac{F}{P + F}\right)$$

The total amount of inorganic N immobilized is given by

$$i = i\left(\frac{P}{P + F}\right) + i\left(\frac{F}{P + F}\right) \tag{5}$$

The amount of labeled inorganic N left in soil where labeled fertilizer has been applied is

$$F - i\left(\frac{F}{P + F}\right)$$

Thus, the quantity of labeled fertilizer N remaining in the soil, and available for plant uptake, is decreased by immobilization, even in the absence of losses from the crop–soil system. The extent of the decrease is determined not only by the amount of N immobilized (i) but also by the quantity of unlabeled inorganic N in soil (P) relative to the quantity of labeled inorganic N added. Where labeled fertilizer has been applied, the quantity of unlabeled inorganic N left in soil after immobilization is

$$P - i\left(\frac{P}{P + F}\right)$$

For the soil inorganic N pool, the difference between the quantity of unlabeled inorganic N remaining in the soil (and presumably available for plant uptake) in the fertilized and unfertilized situations is defined as the Added Nitrogen Interaction (ANI; Jenkinson *et al.*, 1985). It is given by

$$\begin{aligned} \text{ANI} &= P - i\left(\frac{P}{P + F}\right) - (P - i) \\ &= i - i\left(\frac{P}{P + F}\right) \end{aligned}$$

But from Eq. (5),

$$i - i\left(\frac{P}{P + F}\right) = i\left(\frac{F}{P + F}\right)$$

Therefore,

$$\text{ANI} = i\left(\frac{F}{P + F}\right) \qquad (6)$$

(i.e., the ANI is equal to the amount of labeled N immobilized).

In experiments with ^{15}N-labeled fertilizer, it is often observed that uptake of unlabeled N is greater by plants receiving labeled fertilizer than in the unfertilized control. The derivation given here demonstrates that this can occur entirely as a result of "pool substitution," i.e., labeled inorganic N from added fertilizer taking the place of unlabeled inorganic N that would otherwise have been immobilized. The greater the amount of labeled N that is added, the smaller will be the proportion of unlabeled N that is immobilized and the more unlabeled N will remain in the inorganic pool available for uptake by plants. Thus, the observation of increased uptake of unlabeled N is not, in itself, evidence that a true "priming effect" has occurred, in which the addition of fertilizer N causes a greater availability of soil-derived N. It is therefore incorrect to regard the measured uptake of labeled fertilizer N by a crop as an absolute value and intrinsically more reliable than the value obtained from a non-tracer experiment.

In the preceding example, the additional uptake of unlabeled N in the presence of added labeled N (the ANI) is equal to the quantity of labeled N immobilized in soil [Eq. (6)]. Indeed, the measured value for the total quantity of labeled N retained in the plant–soil system is unaffected by pool substitution—It is the relative amounts in plant and soil that are affected. Denitrification can also lead to pool substitution. As with immobilization, the amount of denitrification occurring will often (but not always) be determined by factors other than the amount of nitrate present. However, if an ANI is caused by denitrification, the labeled N responsible for pool substitution is lost from the plant–soil system, in contrast to the situation where the ANI is caused by immobilization.

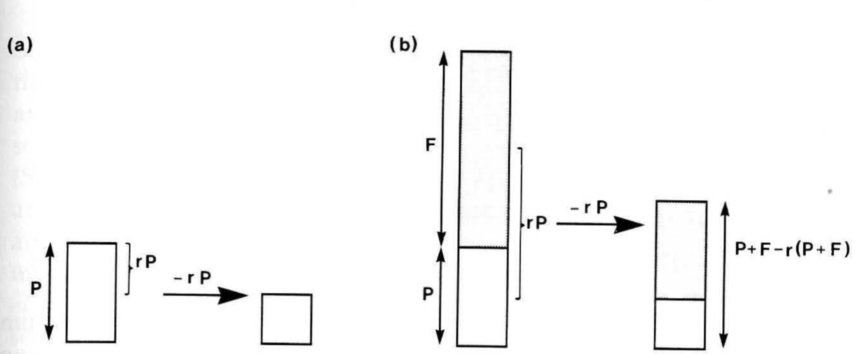

Figure 3 Proportional removal of N (e.g., by leaching) does not cause an ANI. (a) Removal of a proportion r of inorganic N from unamended soil containing a quantity P of inorganic N. (b) Removal of the same proportion r from soil to which an amount F of fertilizer inorganic N has been added. The quantity removed is proportion r of the *total* inorganic N pool.

By contrast to the situation with immobilization and denitrification, removal of N from the soil inorganic pool by leaching does not lead to pool substitution and an ANI. Leaching occurs when a certain proportion of the water in a given soil layer is displaced to a deeper layer, below the rooting depth of plants. The water carries any dissolved N (normally nitrate) with it—The more dissolved N present, the more that will be removed from the layer. The quantity of N lost is a fraction (r) of that present in the layer. In a soil containing an amount P of unlabeled inorganic N in a given layer, the amount remaining after leaching has occurred is $P - rP$ (see Fig. 3a). The quantity remaining in the soil to which an amount F of labeled fertilizer N had been applied is $P + F - r(P + F)$ (see Fig. 3b).

The unlabeled inorganic N remaining in the fertilized treatment is

$$P - r(P + F)\left(\frac{P}{P + F}\right)$$

The additional unlabeled inorganic N left in the amended soil after leaching, less that in the unamended soil, is the ANI, so

$$\begin{aligned}\text{ANI} &= P - r(P + F)\left(\frac{P}{P + F}\right) - (P - rP)\\ &= 0\end{aligned}$$

VII. DETERMINATION OF ^{15}N ABUNDANCE IN SOIL INORGANIC N

Procedures for the determination of ^{15}N/^{14}N isotope ratios in soil ammonium and nitrate are given in Chapter 2. However, it is worth reemphasizing some points. Accurate isotope ratio determination on the inorganic-N fractions in soil involves (a) the quantitative extraction of ammonium and nitrate from the soil, (b) their separation and the conversion of nitrate to ammonium, (c) determination of the N content of both fractions, and (d) isotope ratio determination using either a mass spectrometer or emission spectrometer. In addition to the care normally required in isotope ratio work, two aspects are particularly important with inorganic N. Extracted inorganic-N fractions frequently contain only 20–100 μg N. Reliable methodology must be able to deal with such quantities and to allow correction for isotope dilution resulting from nitrogen introduced into the sample during the preparative stages. The methodology must also permit the separation of the ammonium and nitrate fractions with minimum cross-contamination. Where isotope ratios are being determined on both inorganic-N fractions and, for example, soil microbial biomass, particular care must be taken to avoid cross-contamination (Widmer *et al.*, 1989).

Although there is no universally accepted soil : solution ratio for the extraction of inorganic N with KCl, very narrow ratios (1 : 2) should only be used after checking comparability with more normal ratios (1 : 5). Although the mechanism is not clear, it is apparent that high magnesium levels in the extract slow down the reduction of nitrate by Devarda's alloy and can lead to low recoveries (Clausen *et al.*, 1980).

VIII. DETERMINATION OF ^{15}N ABUNDANCE IN BIOMASS N

A. Background

The soil microbial biomass is a small but dynamic fraction of soil organic matter. It is both the agent of change in soil, being responsible for most of the transformations undergone by nitrogen, and a reservoir of potentially mineralizable nitrogen. Measuring ^{15}N in the biomass can give insight into the fate of labeled fertilizer N or the decomposition of labeled organic materials. It is also necessary in determining the gross rate of immobilization in a soil, as described in Section IX. Two methods for measuring the amount of nitrogen held in the soil microbial biomass have been applied to soil containing labeled N: the chloroform fumigation–incubation method (Shen *et al.*, 1984, using the modified k_N factor given by Jenkinson, 1988) and the chloroform fumigation–extraction method (Brookes *et al.*, 1985, and as modified by Widmer *et al.*, 1989). In both methods, and other modifications of them (e.g., Nicolardot and Chaussod, 1986; Nicolardot *et al.*, 1986), N derived from microbial biomass is measured in a K_2SO_4 soil extract. The following method of analysis involves Kjeldahl digestion of the extract followed by steam distillation.

In determining the ^{15}N content of the soil microbial biomass, it is assumed that the fraction of N from biomass that is analyzed has the same isotopic enrichment as the biomass as a whole. This may not be correct because the N made extractable or decomposable by chloroform is probably derived largely from cytoplasm rather than cell walls. Even if the k_N factor used to calculate total biomass N is correct, it does not follow automatically that the k factor for labeled N is the same. However, it has been shown, with a labeled soil, that the N rendered decomposable by the fumigation–incubation method had the same ^{15}N enrichment as that rendered extractable by the fumigation–extraction method (Brookes *et al.*, 1985).

A third method of estimating N in the soil microbial biomass is the ninhydrin method (Amato and Ladd, 1988), in which ammonium N plus α-amino N in an extract of chloroform-fumigated soil is determined colorimetrically by reaction with ninhydrin. This is a rapid and reliable method of measuring total N in the biomass but, because of the colorimetric method of detection, in which the N is not liberated from the sample, it is not directly applicable to the analysis of ^{15}N in biomass.

B. Reagents

1. Ethanol-free chloroform, $CHCl_3$. Analar grade $CHCl_3$ contains ethanol as a stabilizer, and this must be removed. This is done by shaking with concentrated H_2SO_4 in an extraction funnel three times and with distilled water three to five times followed by drying with anhydrous $CaSO_4$ and distillation, the fraction boiling at 62°C being collected. The purified $CHCl_3$ must be stored in a brown glass bottle, to exclude light, and in a refrigerator, to minimize its decomposition.
2. 0.5 *M* K_2SO_4.
3. Chromium III reagent prepared by dissolving 25 g chromic potassium sulfate [$CrK(SO_4)_2 \cdot 12\ H_2O$,] Analar grade in water, adding 100 ml concentrated sulfuric acid (Analar grade), cooling, and diluting to 500 ml with distilled water.

4. Kjeltab CX tablets containing 5 g K_2SO_4 and 0.5 g $CuSO_4 \cdot 5\ H_2O$ (obtained from Thompson and Capper Ltd., Runcorn, Cheshire, United Kingdom).
5. Sodium hydroxide solution 10 *M*.
6. Boric acid solution 0.25 *M*.
7. Sulphuric acid 0.025 *M* standardized against the amount of ammonia produced by steam distilling a known quantity of Analar grade $(NH_4)_2SO_4$ solution with 10 *M* NaOH.

C. Method

Portions of moist soil (containing between 10 and 50 g oven dry soil) are placed in glass vials in a desiccator that is lined with moist filter paper and contains a beaker of alcohol-free $CHCl_3$ and antibumping granules. The desiccator is evacuated using a water pump until the $CHCl_3$ is boiling vigorously and then closed and left in the dark at 25°C for 24 hr. After this, the portions of soil are transferred to a clean, dry desiccator and the $CHCl_3$ removed by repeated evacuation until it can no longer be detected by smell. Soil is then extracted by shaking with 0.5 *M* K_2SO_4 (4 : 1 solution : soil ratio) for 30 min. The extract is filtered through Whatman No. 42 filter paper and can be stored frozen to await analysis. A 30-ml aliquot of the extract is placed in a 250-ml Kjeldahl digestion tube and gently evaporated almost to dryness on a heating block. Ten milliliters chromium III reagent is added and left at room temperature for at least 2 hr, followed by 3 hr digestion at 360°C with two Kjeltab CX tablets and 40 ml concentrated H_2SO_4. The tube containing the entire cooled contents is cooled further by immersing in ice. Water (20 ml) is added cautiously and the tube allowed to cool again. Ten *M* NaOH (25 ml) is then added slowly, in small portions, care being taken to mix thoroughly after each addition. If these precautions are not taken, the vigorous reaction can become dangerous. In addition, localized concentrations of alkali can cause loss of NH_3. After further cooling, the tube is connected to a distillation apparatus, modified to accept the digestion tube. A further 25 ml 10 *M* NaOH is added cautiously and the contents of the tube are steam-distilled, the distillate being trapped in 5 ml 0.25 *M* H_3BO_3. The total N content of the digest is determined by titration against 0.025 *M* H_2SO_4. For measurement of ^{15}N enrichment, a second digest, prepared from a separate aliquot of K_2SO_4 extract, is treated in the same way as the first and then distilled, the distillate, however, being collected in a small excess of H_2SO_4 instead of H_3BO_3. In principle, isotope ratio analysis can be performed on the distillate collected in H_3BO_3 after titration and acidification, but considerable frothing can occur and cause contamination of the mass spectrometer inlet system. Because of the low concentration of N in the distillate, it is usually necessary to spike with 0.5–1.0 mg unlabeled N as $(NH_4)_2SO_4$ solution prior to drying down for isotope ratio analysis on the mass spectrometer if the Rittenberg procedure is used. This is avoided if ANCA-MS procedures are employed and the dried distillate is transferred directly to a tin cup.

IX. DETERMINATION OF GROSS RATES OF MINERALIZATION AND IMMOBILIZATION

A. Background

Mineralization and immobilization are the release and assimilation respectively, of ammonium, during the decomposition of organic matter by the soil biomass. Despite their importance, both mineralization and immobilization have remained elusive processes, largely because of the difficulty of accurate measurement. Nonisotopic methods can, at best, yield only net mineralization through the nitrogen balance equation

$$\Delta M = \Delta NH_4^+ + \Delta NO_3^- + \Delta plant + loss \qquad (7)$$

where "loss" is N leaving the system via leaching and gaseous emissions. Apart from the inherent uncertainties of determination by difference, with the problem of compounded errors, this procedure relies on determining all the nitrogen lost from the system by leaching, ammonia volatilization, denitrification, and nitrogen oxide evolution during nitrification. Even under laboratory conditions, this is a formidable exercise.

One simplification is to perform laboratory incubations in the absence of plants and in conditions that minimize losses (Stanford, 1982). These have been partially successful as a fertilizer prediction aid to estimate the supply of nitrogen from the soil, but they remain essentially empirical exercises that offer little prospect of any detailed understanding of the processes governing mineralization–immobilization turnover.

At any one time, parts of the biomass are decomposing organic matter with narrow C : N ratios, and mineralizing organic nitrogen, while other parts are decomposing material with wider C : N ratios and immobilizing nitrogen. On this basis, short-term fluctuations in rates of mineralization or immobilization result from inputs of "fresh" organic matter with either narrow or wide C : N ratios. Thus, one approach to predicting mineralization–immobilization turnover in different soils is to relate gross rates of mineralization and immobilization to variations in organic matter composition. This would involve (a) accurately quantifying the flow of C and N into and through the soil biomass in terms of gross rates of mineralization and immobilization; (b) resolving the flux of nitrogen through the biomass into that coming from "young" organic matter, that from current root exudates, that from recycling of the biomass itself, and that from "old" organic matter; (c) quantifying chemical and physical protection of the various organic matter fractions; and (d) characterizing the environmental dependence of the turnover rates of the various fractions.

There is also a clear need to bring together work on the chemical fractionation of organic matter and the more kinetic approaches of the microbiologists. But the first step in this is the determination of gross rates of mineralization and immobilization.

B. Methods for Determining Gross Rates of Mineralization

There are two basic approaches for determining gross rates of mineralization. Those based on analytical equations relating changes in the ^{15}N abundance in a labeled ammonium pool to the rate of mineralization, and simulation models that "hunt" for rate parameters that, when used in simulation models of the soil–plant nitrogen cycle, result in calculated abundances equal to, or close to, those measured.

1. Analytical methods

Kirkham and Bartholomew (1954, 1955) published a set of equations describing the change in the ^{15}N abundance of a labeled ammonium pool receiving nitrogen at natural abundance via mineralization. Perhaps because of their unwieldy formulation, these equations have been little used. More recently, Barraclough *et al.* (1985) and Nishio *et al.* (1985) independently published equations relating secular changes in the ^{15}N abundance of a labeled ammonium pool, to the rate of mineralization and the rate at which the pool size changed. Although not obvious, these later equations are equivalent to the most useful form of Kirkham and Bartholomew's 1955 equations. The derivation of one of them will be detailed here; differences between it and the other versions of Kirkham and Bartholomew's equations will be indicated.

Consider a soil ammonium pool to which, at $t = 0$, a quantity of labeled ammonium is added. If the added label mixes with the indigenous soil ammonium, and there is no preferential utilization of either label or unlabeled ammonium, processes that consume ammonium will remove labeled and unlabeled ammonium from the pool in proportion to the amounts present. Under these conditions, *they will not in themselves alter the ^{15}N abundance in the pool.* This assumption of proportional exploitation (see Fig. 4) is central to mathematical treatments of mineralization. If it is valid, the only process altering the abundance of the pool is mineralization; it reduces the ^{15}N abundance by introducing unlabeled N into the pool. The faster the rate of mineralization, the more rapid the decline in pool abundance. In addition, although in themselves they do not alter the pool abundance, the processes consuming ammonium will modify the effect of a given rate of mineralization because they remove a mixture of labeled and unlabeled N that is replaced by unlabeled. The process of mineralization-induced pool dilution is depicted in Fig. 5.

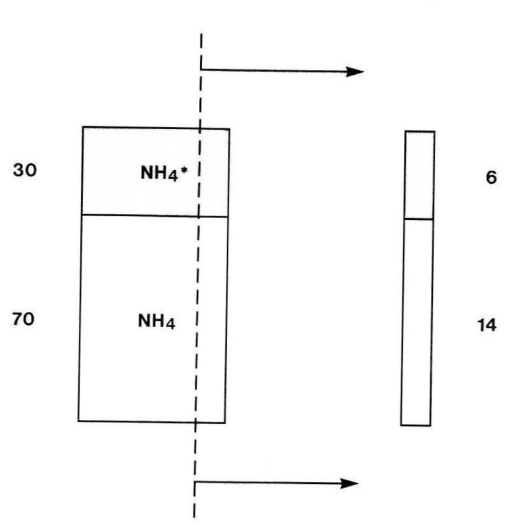

Figure 4 The principle of proportional exploitation of an ammonium pool containing labeled and unlabeled N.

Translating these qualitative relations into useful quantitative ones requires four assumptions:

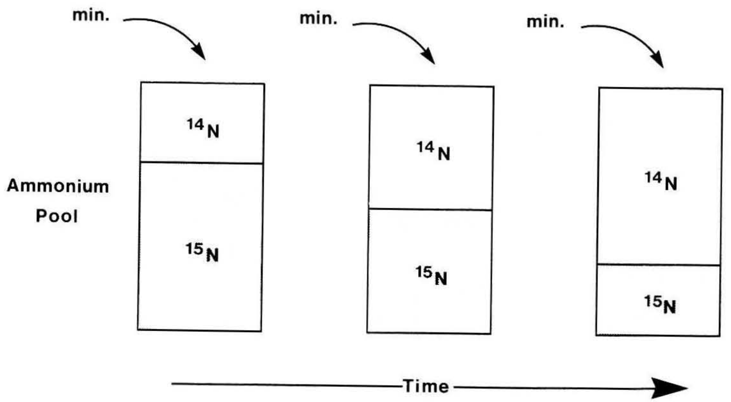

Figure 5 Dilution of an ammonium pool with unlabeled N derived from mineralization (min).

1. Processes such as plant uptake and nitrification do not discriminate between ^{14}N and ^{15}N.
2. Added label mixes with indigenous soil ammonium such that labeled and unlabeled N are used in proportion to the relative amounts present.
3. Over the experimental period, all rate processes can be described by zero-order kinetics.
4. Labeled nitrogen immobilized over the experimental period is not remineralized.

With these assumptions, it is possible to derive Eq. (8) for the excess ^{15}N enrichment in the soil ammonium pool (Barraclough *et al.*, 1985):

$$A_t^* = A_0^*/(1 + \theta t/A_0)^{m/\theta} \tag{8}$$

where A is the size of the ammonium pool, t is time, and m is the rate of mineralization. Superscript * indicates atom% excess, subscripts 0 and t indicate $t = 0$ and $t = t$; θ, the rate at which the ammonium pool changes size, is given by

$$\theta = m - i - l - n - u_a \tag{9}$$

where u_a is the rate of plant uptake from the ammonium pool, n is the rate of nitrification, i is the rate of ammonium immobilization in the biomass, and l is the rate at which ammonium is lost from the system.

In experimental terms, θ is given by

$$\theta = (A_t - A_0)/t \tag{10}$$

Thus, performing a ^{15}N tracer experiment in which the size and ^{15}N abundance of the ammonium pool are measured at two times, $t = 0$ and $t = t$, gives all the information required to solve Eq. (8) for m, the rate of mineralization. An example is given below.

Example calculation
In a field experiment, 22.5 kg ammonium N with a ^{15}N atom% excess of 5.217 was added to a grassland sward. Samples were taken immediately following application ($t = 0$) and after 18 days ($t = t$). The following results were obtained:

Time (days)	NH_4 Pool Size (kg N ha^{-1})	NH_4 Atom% Excess
0	28.0	4.1955
18	12.3	0.565

This gives $\theta = (12.3 - 28.0)/18 = -0.870$ kg N ha^{-1} day^{-1}, which in Eq. (8) gives

$$0.565 = 4.1955/[1 + (-0.870*18/28.0)]^{\frac{m}{-0.870}}$$

Solved for m, this gives $m = 2.129$ kg N ha^{-1} day^{-1}.

Chalk *et al.* (1990) determined rates of mineralization using both Eq. (8) and Kirkham and Bartholomew's extended equation. Over the time intervals used (12 days), the two methods gave very similar results.

2. Numerical Methods

Analytical methods such as the preceding ones are elegant and relatively easy to use, but they are restricted in their applicability by the validity of the assumptions used to derive the equations. The two most restrictive assumptions concern the zero-order kinetics and the lack of remineralization. In their 1955 paper, Kirkham and Bartholomew derive an equation for mineralization and immobilization in which the rates are assumed to be proportional to the size of the inorganic and organic pools, respectively. However, as Bjarnason (1988) points out, there is little biological basis for this assumption. But mineralization and immobilization rates will change over time in response to carbon or nitrogen availability and environmental changes, and the time window over which the various rate processes can be approximated by zero-order kinetics is probably quite short, perhaps 7–14 days. Where it is required to follow transformations over extended periods, therefore, there is little option but to use simulation modeling. Similarly, remineralization of labeled nitrogen will occur. The time window over which it can be assumed to be negligible is also probably of the order of 7–14 days (Bjarnason, 1988). Again, simulation modeling is the best way to overcome such a restriction. To date, one model, MIMIC, has been published (Bjarnason, 1988), although other workers have used simulation modeling to determine mineralization rates (Shearer *et al.*, 1974; Paul and Juma, 1981; Tiedje *et al.*, 1981; Hart *et al.*, 1986; Myrold and Tiedje, 1986).

Bjarnason's model uses a set of simultaneous first-order differential equations to describe changes in the labeled and unlabeled parts of the organic, ammonium, and nitrate pools in soil. Corresponding expressions for the unlabeled nitrogen are given. These equations are solved to yield the amounts of labeled and unlabeled N in each pool as a function of time. By combining this simulation model with an optimization procedure, rates of mineralization and immobilization can be estimated by searching for rate parameters that result in pool ^{15}N abundances equal to those measured. The technique is powerful and flexible. There is no requirement to set zero-order kinetics; any time dependence can be built into the differential equations. Remineralization of labeled N can be accommodated. In Bjarnason's work, remineralization was apparent 14 days after application of labeled ammonium and 28 days after the addition of labeled nitrate (Bjarnason, 1988).

Simulation-parameter optimization procedures are a powerful and flexible tool that deserve a wider audience. They are underemployed, partly because of concern over a perceived complexity in setting them up. With the introduction of good, commercially available packages to perform the simulation and optimization, this concern is largely unjustified for any researcher with modest programming ability. Bjarnason used a software package called MODAID in his work, but it is not widely available. Details and availability of a simulation–optimization program called DOMINUS (Determination of Mineralization, Immobilization and Nitrification Using Simulation), for the interpretation of ^{15}N tracer experiments, can be obtained from the second author of this chapter. The program requires a 286- or 386-based PC fitted with a math coprocessor.

C. Methods for Determining Gross Rates of Immobilization

Immobilization is defined here as the assimilation of inorganic N into the soil microbial biomass and its subsequent incorporation into other fractions of the soil organic matter. It does not include assimilation into root tissue. As with mineralization, there is the direct analytical (in the mathematical sense) method in which the amount of label recovered in the biomass is interpreted in terms of the rate of gross immobilization through simple equations (Kirkham and Bartholomew, 1954, 1955; Shen *et al.*, 1984). The alternative is to use simulation modeling (Paul and Juma, 1981; Bjarnason, 1988).

1. Analytical Methods

Kirkham and Bartholomew (1954) give the following equation for determining immobilization, $i\Delta t$, in ^{15}N tracer studies:

$$i\Delta t = (A_0 - A_t)\cdot\ln (A_0{}^*/A_t{}^*)/\ln (A_0/A_t) \tag{11}$$

Because Kirkham and Bartholomew's representation of the soil nitrogen cycle only included two pools, the inorganic and organic, this equation will yield the sum of immobilization and any other processes that consume ammonium. Thus, to determine immobilization accurately, nitrification and any ammonium loss process need to be measured and offset against the calculated rate of immobilization. Shen *et al.* (1984) used the arithmetic mean of the ammonium pool ^{15}N abundance over the interval, $[^{15}A]$, and the amount of ^{15}N in the organic pool at the end of the interval, $O_t{}'$, to give

$$i\Delta t = O_t{}'\cdot([A]/[^{15}A]) \tag{12}$$

This is more robust than Eq. (11) in that it will correctly estimate immobilization even when other processes are consuming ammonium. But the use of simple arithmetic means restricts the time interval over which it can be used. It also assumes that all immobilization occurs from the ammonium pool. In practice, some labeled ammonium will be nitrified and subsequently immobilized from the nitrate pool. And finally there is the question of how the ^{15}N abundance of the organic pool is defined: the whole organic matter or just the biomass?

In the short term, most immobilized N will be in the biomass. Thus, it is sensible to extract the biomass and determine its ^{15}N abundance. This avoids the problem of the large background of organic N diluting the label. Over longer periods (>28 days) cycling of the biomass itself and incorporation of label into the humified fractions of the soil organic matter will become significant. Under these conditions, determinations of isotope ratios should be performed on the whole organic matter.

The problem of simple arithmetic means can be avoided by using correct averaging procedures. If the ^{15}N abundance of a labeled ammonium pool can be described by Eq. (8), then the mean abundance over the interval is given by (Barraclough *et al.*, 1985)

$$\int A^*\, dt/(t_2 - t_1)$$

which gives

$$[A^*] = \frac{1}{t_2 - t_1} {}_{t_1}^{t_2}\left(\frac{A_0{}^{m/\Theta}\cdot A_0{}^*\cdot(A_0 + \Theta t)(1 - m/\Theta)}{(\Theta - m)}\right) \tag{13}$$

where square brackets indicate mean pool abundances (Barraclough, 1991a,b). When $\Theta = m$, an alternative expression should be used. If it is required to estimate the immobilization of both ammonium and nitrate, two tracer experiments should be performed: one in which both pools are labeled and a second in which only the nitrate pool is labeled. The ^{15}N abundances of both the ammonium and nitrate pools should be determined at the beginning and end of the experimental period, together with the ^{15}N abundance of the soil microbial biomass (or the whole organic matter if a longer interval is used). Equation (13) applies to either an ammonium pool labeled at $t = 0$ and receiving nitrogen at natural abundance via mineralization, or a nitrate pool labeled at $t = 0$ and receiving unlabeled nitrogen via nitrification, or to the whole inorganic N pool (see Barraclough, 1991a,b). Thus, the mean pool abundances of the nitrate pool in the labeled nitrate experiment can be calculated. The immobilization from the nitrate pool is then given by

$$i\Delta t = O\cdot(O^*)/[N^*], \tag{14}$$

where O refers to either the biomass or the whole soil organic matter and $[N^*]$ is the mean excess abundance of the nitrate pool. Using the nitrate pool separately avoids double-counting label originating in the ammonium pool but being immobilized, after nitrification, from the nitrate pool. The same calculation is carried out for the experiment in which both the pools are labeled. In this case, the mean pool abundance is that of the whole inorganic N pool. An analogous expression to Eq. (14) is then used to determine immobilization from the whole inorganic N pool; immobilization from the ammonium pool follows by difference. If only overall immobilization is required, only the double-labeled experiment need be performed.

This procedure for determining immobilization is not as robust as that for mineralization, and how reliable it is remains to be seen.

2. Numerical Methods

Numerical methods are similar to the procedures for determining mineralization. In this case, the ^{15}N abundance of the organic matter pool (or the microbial biomass) must be simulated. The optimization procedure then uses the agreement between the predicted and observed abundance in the organic matter as the criterion for parameter estimation. The same reservations noted in the mineralization procedures apply here.

D. Conclusions

Although some of the procedures described earlier are not new, only relatively recently have they been used to determine the gross rates of nitrogen transformations. There is still insufficient experimental data to evaluate them critically, and this must be one priority in ^{15}N soil tracer studies. If they prove reliable, they offer a real prospect of unraveling the complexities of the soil–plant nitrogen cycle.

X. GENERAL CONCLUSIONS

The use of ^{15}N makes it possible to explore, quantify, and understand the processes of mineralization and assimilation in ways that are impossible without using a tracer. The combination of mathematical modeling and experimental approaches is particularly powerful. The development of new analytical techniques, especially ANCA-MS, has made the use of ^{15}N less laborious and more easily applicable. Despite the exciting developments, it is appropriate to end with two cautions. First, attention to detail is necessary at every stage of an experiment with ^{15}N—field operations, laboratory methodology, sample preparation, and analysis. Second, when interpreting results, the assumptions that are made should be critically evaluated for each particular situation.

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9

Mineralization and Assimilation in Aquatic, Sediment, and Wetland Systems

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I. INTRODUCTION

Knowledge of the nitrogen cycle in marine and fresh waters has been advanced tremendously by application of tracer techniques, especially those employing ^{15}N. Why use tracer techniques? Most importantly, although changes in the concentration of a particular nutrient with time can provide information on the net balance of production or use of that nutrient, such changes are often the result of multiple metabolic reactions. Tracer procedures allow us to dissect the individual processes contributing to the net flux. Additionally, in oceanic and oligotrophic freshwater systems, the concentrations of nitrogenous nutrients often border on the analytical limit of detection for wet chemistry and, for all practical purposes, remain quite constant. Thus, concentrations alone give no information about the rates of uptake and regeneration when they are in close balance. Tracer and isotope dilution techniques allow us to discern otherwise imperceptible rates of uptake and regeneration of nitrogen.

In general, a nitrogen tracer experiment begins with the addition of a known quantity of a ^{15}N isotopically labeled compound. The movement of that tracer into different particulate or dissolved pools is monitored with time. Tracer movement is monitored by the measurement of the change in isotopic enrichment (ratio of ^{15}N/^{14}N) in the substrate and particulate fractions. Thus, for example, in ^{15}N uptake studies, one measures the

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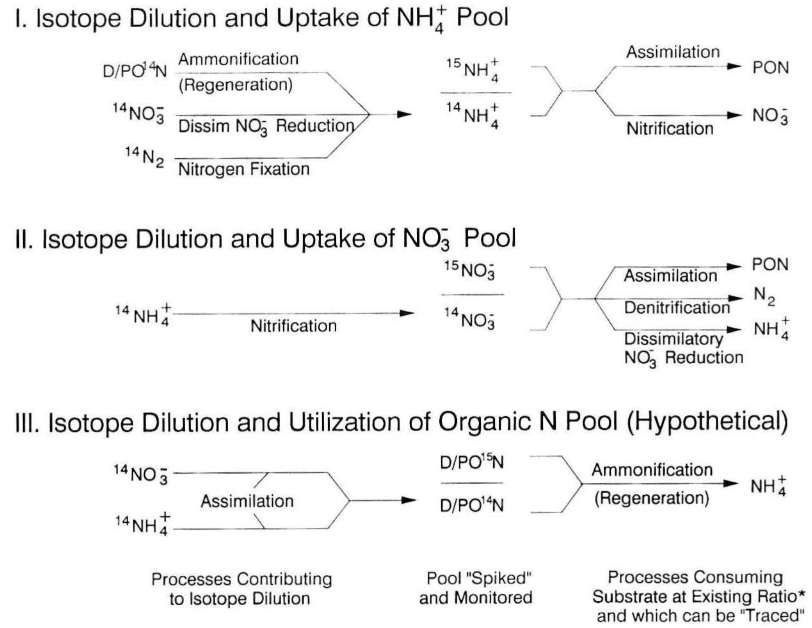
I. Isotope Dilution and Uptake of NH_4^+ Pool
D/PO^{14}N
Ammonification
(Regeneration)
$^{14}NO_3^-$
Dissim NO_3^- Reduction
$^{14}N_2$
Nitrogen Fixation
$^{15}NH_4^+$
$^{14}NH_4^+$
Assimilation
PON
Nitrification
NO_3^-
II. Isotope Dilution and Uptake of NO_3^- Pool
$^{14}NH_4^+$
Nitrification
$^{15}NO_3^-$
$^{14}NO_3^-$
Assimilation
PON
Denitrification
N_2
Dissimilatory NO_3^- Reduction
NH_4^+
III. Isotope Dilution and Utilization of Organic N Pool (Hypothetical)
$^{14}NO_3^-$
$^{14}NH_4^+$
Assimilation
D/PO^{15}N
D/PO^{14}N
Ammonification
(Regeneration)
NH_4^+
Processes Contributing to Isotope Dilution
Pool "Spiked" and Monitored
Processes Consuming Substrate at Existing Ratio* and which can be "Traced"

Figure 1 Schematic diagram of the various processes in the nitrogen cycle, which can be traced using techniques of isotope dilution or isotope enrichment. (I) Processes conveniently traced when the dissolved NH_4^+ pool is initially enriched with a known quantity of $^{15}NH_4^+$; (II) as for (I), except for NO_3^-; (III) as for (I), and (II), except for organic nitrogen. See details in text.

progressive increase in isotopic enrichment of the particulate fraction as a function of time as the substrate is utilized. In isotope dilution studies, one initially enriches a dissolved nitrogen pool and monitors the progressive decrease in enrichment with time due to release of the unlabeled form of that nitrogen compound. One can also label the dissolved fraction with a ^{15}N-labeled nitrogen compound and monitor the production of another labeled dissolved nitrogen product. Such would be the case in nitrification studies, where one adds labeled NH_4^+ and follows the production of ^{15}N-labeled NO_3^-. The use of ^{15}N in measuring these varied pathways is shown in Fig. 1.

In the past decade, there has been a proliferation of measurements taking advantage of nitrogen tracer techniques. These techniques have allowed the estimation of the rates of uptake and transformation of all the major organic and inorganic species of nitrogen in a broad range of fresh- and seawater systems. They have yielded estimates of mineralization, regeneration, and nitrification in water and sediments. Isotopic procedures have been used to determine the physiological preferences of phytoplankton for different forms of nitrogen and to assess the nutritional status of phytoplankton. With increasing sophistication of these methods has come a greater awareness for the problems and pitfalls of the techniques. Early measurements using ^{15}N in uptake experiments were, from our current perspective, very simplistically applied. For example, there was little recognition that concurrent processes, such as regeneration, could compromise a tracer uptake measurement. Our goal in this chapter is to review methods for determination of uptake and regeneration (release and mobilization) of the major forms of nitrogen in the water column and sediments. While we emphasize the problems and pitfalls, we do not intend to discourage the use of these tools but, rather, aim to provide a rigorous foundation for their application. No other current techniques can yield comparable data on nitrogen flux. Other recent reviews of ^{15}N methodology and applications in coastal and marine waters (Harrison, 1983; Dugdale and Wilkerson, 1986; Glibert, 1988; Paasche, 1988) deal with some additional aspects of the interpretation of these types of experiments.

II. METHOD 1: AQUATIC/WATER COLUMN STUDIES

There is a great deal of variation in the specific ways ^{15}N isotopic techniques are applied by individual investigators. One approach may be appropriate to one set of conditions or questions, but it may be entirely inappropriate in another situation. Different experimental designs may lead to dissimilar results and conclusions. Thus, we cannot describe a "standard" procedure but, instead, discuss a range of approaches and their implications.

A. Measurement of Uptake and Assimilation

1. Incubation Protocols

A tracer experiment begins with the water sample being dispensed into suitable experimental containers. The sample is often prescreened, using Nitex netting or filters, to exclude all organisms larger than a desired size. In contrast to ^{14}C productivity experiments, in experiments on NH_4^+ uptake and release by size class, the size fractionation must be done prior to tracer inoculation (discussion of tracer additions follows) and incubation, because regeneration will result in a change in the isotopic composition of the NH_4^+ pool during incubation. The relative contribution of each size class to this change cannot be determined if the size classes are not separated beforehand. It is important to note, however, that total NH_4^+ regeneration in a natural food web may be different from that measured in component size fractions, and the extent of this difference is a nonlinear function of zooplankton density and/or multiple trophic interactions (Glibert *et al.*, 1992).

Types of incubation containers have been discussed by Carpenter and Lively (1980) and Fitzwater *et al.* (1982); polycarbonate is usually preferred over glass because of potential contamination by trace metals. If a time course is being conducted, the choice must be made between adding tracer to one large bottle from which aliquots can be removed at intervals and adding tracer to replicate smaller bottles, filtering each one completely at each time point. The advantage of the first approach is the simplicity of dealing with only one bottle; when replicate bottles are prepared, the tracer addition must be very precise to ensure reproducibility, and incubation conditions for each bottle must be identical throughout the period of incubation. The disadvantage of the single bottle approach is that it must be well mixed before each subsample is removed, and some organisms are particularly sensitive to shaking and agitation. Smith and Horner (1981) recommend that replicate bottles be used when radioactive isotopes are involved.

The decision of how long to allow an incubation to proceed is a difficult one. Ideally, time courses with subsamples ranging from a few minutes to several hours should be conducted, and the rate calculated from the "linear" portion of the uptake curve. In practice, handling this many samples is not always possible. An incubation period should be selected that is sufficiently short so that substrate will not become significantly depleted and the quantity and composition of the biomass will not have changed (Venrick *et al.*, 1977; Collos, 1987). Depending on length of incubation, one may be measuring different cellular metabolic processes (Glibert and Goldman, 1981; Wheeler *et al.*, 1982; Zehr *et al.*, 1988). For example, while transport across the cell membrane occurs in the first seconds to minutes, with time, more and more of the nitrogen is assimilated into amino acids and protein. The amount of nitrogen assimilated into proteinaceous material can be determined at the end of the incubation by rinsing the filter with ~25 ml of cold 5% trichloroacetic acid; this will precipitate the proteinaceous material (Glibert and McCarthy, 1984).

2. Determining Tracer Addition

In initiating a tracer experiment to determine the rate of nitrogen uptake by natural plankton in a water sample, it is important to evaluate carefully the amount of tracer to be added. This is especially true in oligotrophic waters, where the availability of nitrogenous nutrients and the plankton biomass are often extremely low. Thus, a tracer addition can represent an enrichment above ambient and may perturb the nutritional status of the cells. Two questions must therefore be asked before starting any experiment: First, what is the ambient concentration of the nitrogen nutrient of interest (NO_3^-, NH_4^+, urea, etc.), and, second, what is the process of interest—*in situ* rates of uptake or maximum, potential rates of uptake?

If we assume that the water being studied has low ambient nitrogen, and *in situ* rates of uptake are being addressed, then the smallest possible addition of ^{15}N-labeled substrate is desirable. Typically this is 10% of ambient (Dugdale and Goering, 1967), or, if ambient is below the limit for concentration detection, an addition is made that is equal to the lower limit of detection. At these low levels of tracer addition, the usual source of error in the resulting rate will be in the estimate of substrate concentration and generally not in the determination of isotope enrichment of the particulate pool. However, if an addition much less than 10% of ambient is made, extreme care must be taken to analyze the atom% of the particulate fraction precisely. Inflated rates of uptake can result in this case due to the fact that the denominator in the calculation is extremely small (Glibert and Bronk, unpublished data).

Because it has been demonstrated (e.g., Eppley and Coatsworth, 1968) that the specific rate or velocity (V) of uptake of nitrogen can be related by a rectangular hyperbolic function to the substrate concentration (analogous to Michaelis–Menten kinetics), it follows that as increasingly larger amounts of labeled substrate are added, increasingly higher rates of uptake will be measured until the maximal specific uptake rate or velocity (V_{max}) has been attained. Hence, even a 10% increase in the ambient pool should stimulate the rate of uptake, if the original concentration falls on the proportional segment of the curve. Depending on the slope, a 10% increase in concentration could stimulate uptake by >10%. From a practical standpoint, it is often impossible to conduct the number of experiments necessary to fully define the saturation curve (and its parameters K_s, V_{max}), but experiments at more than one concentration are often desirable to gain insight into the nutritional and physiological condition of the cells (e.g., Wheeler *et al.*, 1982; Glibert and McCarthy, 1984).

There are situations where the determination of the appropriate amount of ^{15}N to add is not as straightforward. One such situation occurs when the ambient concentration of the nitrogen substrate is near the detection limit, but the biomass is relatively high. This might be observed, for example, at the end of a phytoplankton bloom. In this case, if an addition is made equal to the analytical limit of detection for concentration measurements, it is highly likely that substrate depletion will rapidly occur, perhaps in a matter of minutes. In such a case, incubations should be kept very short and a higher addition (on the order of 10% of the nitrogen biomass) might be considered.

3. Sample Harvesting

When an incubation is terminated, the sample must be filtered. For all uptake and assimilation determinations, the filter is retained for mass or emission spectrographic analysis and, if regeneration is simultaneously being determined, the filtrate is also retained. The choice of filter is an important one. It must not contain nitrogen, which will contaminate the analysis. Commercial filters typically contain unacceptable levels of nitrogen and must, at a minimum, be combusted before use (2 hr, 500°C). The filter most widely employed for routine use is the Whatman GF/F. This filter, however, has a nominal pore size of 0.7 μm and, thus, allows a considerable amount of bacteria and small phytoplankton to pass through. To obtain better retention, a double thickness of the GF/F filter can be used. Many other currently available filters either have unacceptably slow filtration rates or are of a carbon matrix that may create contaminating gaseous by-products upon combustion. The Anopore™ Inorganic Membrane filter (Alltech Associates, Inc.) shows promise for some applications, with its improved flow characteristics. Before combusting this filter, the edging material can be easily peeled off to reduce the blank.

It is not essential that the filtration for ^{15}N isotopic analysis be quantitative. In practical terms, this means that if the filter begins to clog, and sufficient material has been filtered to satisfy the mass requirements of the mass or emission spectrometer (see Section IV.A.2.b), the filtration can then be terminated. The only exception to this generalization is if the same sample is being used for a determination of the particulate nitrogen content of the sample (i.e., from the N_2 gas pressure in the inlet line of the mass spectrometer); then, quantitative filtration is imperative. Filters are typically rinsed with 25–50 ml filtered seawater prior to turning off the filtration vacuum, and it is important that this rinse be added before the filter with sample is exposed to air (Goldman and Dennett, 1985). Thus, as the last few milliliters of sample are filtering, the rinse is added. Then, the filter is removed, placed in a plastic or precombusted aluminum foil envelope, and frozen or dried.

4. Preparation of Sample for Isotopic Analysis

Chapters 2 and 3 in this volume, and Fiedler and Proksch (1975), have covered fairly extensively the steps required to prepare samples for mass or emission spectrometry. The automated sample preparation system described by Owens (1988), which couples an elemental analyzer to a triple collector isotope ratio mass spectrometer, is an exciting development and allows for the efficient processing of many more samples than can be manually prepared.

B. Measurement of Regeneration and Release

1. Incubation Protocols

The mechanics of incubation for a regeneration or release experiment are essentially the same as those for an uptake experiment. Indeed, for the most accurate work, they should be done on identical samples (see Fig. 1).

2. Determining Tracer Addition

The quantity of isotopically labeled tracer that is added to the experimental vessel depends on whether the experimental design involves the measurement of (a) the degree of isotope dilution of the same nitrogen form (i.e., NH_4^+) as the added ^{15}N label, (b) the production of a nitrogen form that is different from the one isotopically labeled initially, or (c) the change in two labeled forms of the same compound, as is the case for urea. We treat each of these cases individually below.

a. For determination of isotope dilution of the added isotopic tracer Simply put, the isotope dilution technique, which is most commonly applied to the study of NH_4^+ uptake and regeneration, involves the addition of $^{15}NH_4^+$ to a water sample and, after some period of incubation, measuring the ^{15}N incorporated into the particulate material (= uptake) and coincident dilution of the $^{15}NH_4^+$ by $^{14}NH_4^+$ in the dissolved pool (= regeneration). The experiment should proceed over a period of time that is short relative to the time required for isotopic equilibration or for regeneration of added label to occur. By simultaneously measuring the change in concentration in the NH_4^+ pool, the incorporation into particulate material, and the change in the isotopic ratio of the NH_4^+ pool, one can calculate the rate of uptake and release (see Section IV.C).

The amount of isotope dilution will depend on the biological rate of regeneration but will also depend, from a methodological viewpoint, on the amount of added isotope at the start of the experiment relative to the ambient concentration. For example, in oligotrophic seawater, where the ambient concentration of NH_4^+ is often below detection limits for standard colorimetric methods, a "trace" $^{15}NH_4^+$ addition will elevate the isotope enrichment of the NH_4^+ pool on the order of 50–100% (ambient of 0–0.03 μM plus $^{15}NH_4^+$ of 0.03 μM). A regeneration or production rate of NH_4^+ of 0.03 μM hr^{-1} will reduce the initial ^{15}N isotope enrichment by about half over a 1-hr period (assuming uptake approximately equal to regeneration). This change in isotope enrichment should be easy to resolve even with an emission spectrometer. The situation in which a saturating addition (say 10.0 μM) is made to the same oligotrophic seawater having the same regeneration rate is very different. Here the amount of NH_4^+ release during a 1-hr period would have little effect on the isotope enrichment of the NH_4^+ pool (the production of 0.03 μM $^{14}NH_{4+}$ is a small fraction of the starting concentration). Thus, to measure a regeneration rate of NH_4^+, the isotope addition should be such that the change in isotope enrichment is maximized. Otherwise, one might erroneously conclude that regeneration did not occur, when in fact the experiment was poorly designed and a small change against a large background could simply not be observed.

Isotope dilution may also be employed in studies of dissolved organic nitrogen (DON; Fig. 1) and preliminary experiments carried out in the author's (P.M.G.) laboratory show promise for this technique for assessing total DON uptake and regeneration (D. A. Bronk, unpublished data). In addition, isotope dilution techniques could be applied for tracing specific organic nitrogen pools, such as amino acids or methylamines. An amino acid pool could be spiked with a ^{15}N labeled compound and the total pool of that amino acid monitored with time by high-pressure liquid chromatography (HPLC; Lindroth and Mopper, 1979) while collecting discrete samples for isotope enrichment. However, the mass requirements of mass and emission spectrometry likely exceed that obtainable from analytical HPLC and may require concentration of samples. Fuhrman (1987) has taken a combined HPLC–radioisotope approach in studies of ^{14}C amino acids where samples of separated amino acids eluting from the HPLC are assessed for radioactivity in a flow-through detector or by liquid scintillation counting after collection. While the fate of carbon and nitrogen derived from amino acids may be different (Zehr *et al.*, 1985), there is no *a priori* reason to expect that rates of isotope dilution as determined by ^{15}N or ^{14}C substrates will be different. The use of ^{14}C is far more sensitive and convenient.

b. For determination of production of a soluble labeled compound different from the added isotopic tracer To account fully for the complexity of the nitrogen cycle, it is desirable in many systems to measure the rate of transformation of one form of nitrogen to another (Fig. 1). These experiments begin with the addition of ^{15}N-labeled substrate, say in the form of NH_4^+, and the production of ^{15}N-labeled product (e.g., NO_3^-) is monitored with time. The determination of the appropriate ^{15}N tracer addition for these types of experiments shares many of the problems and trade-offs discussed earlier for uptake and regeneration experiments. Relatively large or saturating additions will facilitate the measurement of isotope in the product simply because the isotope signal will be larger. However, when

ambient concentrations of the labeled substrate are initially low, a large addition may artificially enhance the rate of transport, may hasten the filling of internal cellular pools of nitrogen, and may hasten the production of product compound. A trace addition will yield more realistic values of the *in situ* rates of nitrogen transformation although, if the rates are low, a sensitive mass spectrometer will be required to detect isotopic transfer to the product pool. When ambient concentrations of the labeled substrate and product are sufficiently high, a 10% addition may be made and detected in the product (if that transformation is indeed occurring) with less difficulty than when concentrations border on the detection limit.

c. For determination of regeneration and uptake of the same substrate when two tracers must be used Phytoplankton physiologists and oceanographers have for many years recognized that urea regeneration is significant, but direct isotopic measurements of this process, in a manner analogous to NH_4^+ isotope dilution measurements, have been extremely difficult to make. The main reason is that the urea must be converted to NH_4^+ before isotope analysis, and contamination problems are too great to do this with large volumes of water with sufficient accuracy. A method developed by Hansell and Goering (1989) builds upon basic isotope dilution principles but involves the use of two isotopes, ^{14}C-urea and ^{15}N-urea. ^{15}N-labeled urea is used for the determination of the rate of uptake following the procedures described earlier. A second tracer, ^{14}C-urea, is used, either in the same incubation vessel (Hansell and Goering, 1989) or in a parallel incubation vessel to determine the isotope dilution (decrease in specific activity) due to the excretion and release of urea. Thus, the strength of using ^{15}N- over ^{14}C-urea in uptake studies is maintained (^{14}C-urea uptake yields an estimate of metabolism, not total uptake because some of the ^{14}C can be respired as $^{14}CO_2$) and the ease of monitoring changes in specific activity as an indicator of urea production is exploited. The seawater must be bubbled to drive off any released $^{14}CO_2$ before assaying for radioactivity. We recommend the use of parallel incubations, each inoculated with identical urea concentrations but different isotopes. The necessary measurements of concentrations of urea can be done on the sample labeled with ^{15}N-urea, thus avoiding radioactive contamination of glassware and instrumentation. The obvious assumption involved in the use of parallel incubations is that rates of uptake and release are identical in the two bottles. Calculations are described in Section IV.C.1.

3. Sample Harvesting

In contrast to studies designed solely for the measurement of uptake of nitrogenous compounds, where the final sample for analysis is in particulate

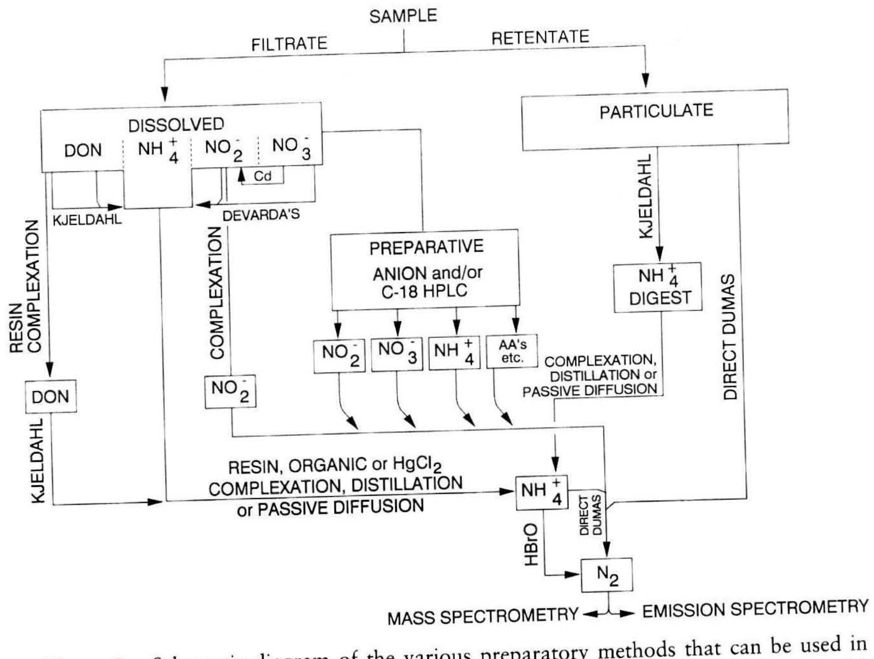

Figure 2. Schematic diagram of the various preparatory methods that can be used in recovering either the dissolved nitrogen species of interest or the particulate nitrogen material for subsequent mass or emission spectrometric methods.

form, in regeneration and release experiments, the dissolved nitrogen species of interest must be collected and separated. These analytical techniques are described here.

a. NH$_4^+$ There are several methods for collecting NH_4^+ for isotopic analysis from freshwaters and seawaters (Fig. 2). The first method developed for this purpose is that of steam distillation (cf. Bremner and Edwards, 1965; Bremner and Keeney, 1965). Similar methods utilizing vacuum distillation (Harrison, 1978; Glibert *et al.*, 1982) have more recently been applied. Commercially available NH_4^+ distillation apparatus (e.g., Corning 3340) are adequate and available in different sizes to accommodate different sample volumes. Distillation techniques begin with an addition of base, either NaOH or a saturated solution of MgO, to a measured volume of sample in a distillation flask in order to raise the pH of a sample to 10 or above. The sample is heated using a heating mantle, and the liberated NH_3 is collected in a small quantity (e.g., 10 ml) of dilute acid in an Erlenmeyer flask. The distillate must be collected directly in the acid solution and not

dripped in because significant loss to the walls of the collecting flask can occur. For mass spectrometry, an HCl solution of nominally 0.0024 *N* is used, because repeated processing of much more acidic samples may lead to corrosion of the fittings on the inlet line or in the mass spectrometer itself. The distillation proceeds until 20–25% of the original sample volume is collected in the acid trap. If covered with parafilm, the distillate may be stored for ca. 1 day at room temperature without problems. For longer storage it is recommended that the distillate be frozen, or evaporated to a volume of 10–15 ml and decanted into a tightly capped scintillation vial. In this form, the sample is stable indefinitely. The sample is subsequently prepared for mass or emission spectrometry as described in Section II.B.4.

A number of alternative methods to distillation are available for collecting NH_4^+ for isotopic analysis. The first, based on a nephelometric determination of ammonia in blood (Graves, 1915), involves precipitation of NH_3 using an alkaline solution of mercuric chloride (Fisher and Morrissey, 1985). This method has been successfully applied to freshwaters and brackish waters but requires some modifications for use in seawater. An obvious disadvantage of this technique is the fact that large quantities of caustic poisonous waste are generated during sample processing. A second method, described in detail by Dudek *et al.* (1986), involves conversion of NH_4^+ to indophenol using a modification of the phenol–hypochlorite reaction (Solorzano, 1969) followed by extraction of the indophenol into dichloromethane, and evaporation and drying of the sample. The rate of indophenol conversion is enhanced by using sodium aquopentacyanoferrate (Patton and Crouch, 1977; Dudek *et al.*, 1986). As earlier, these reagents are poisonous; hoods must be used. Additionally, a significant nitrogen blank is introduced with the reagents, but this appears to be a constant.

A third approach for extraction of NH_4^+ from natural waters has been described by Selmer and Sörensson (1986). This method, like the Dudek *et al.* (1986) method, begins with the conversion of the NH_4^+ to indophenol; at that point, the compound is concentrated onto an octadecylsilane (C18) column (commercially available Sep-Pak cartridge, Waters Associates, Milford, Massachusetts) and eluted with alkaline 10% methanol–water solvent. The sample is then evaporated and dried onto a Whatman GF/F filter. Of the extraction methods, this is the least hazardous; however, two problems require critical evaluation by each investigator. First, the recovery of NH_4^+ is low, on the order of 28% (Selmer and Sörensson, 1986), which, as described later, may have ramifications in the final precision of the ^{15}N atom%. There is also a significant reagent blank.

A fourth approach (Kristiansen and Paasche, 1989) utilizes direct diffusion. A water sample is placed in a new acid- and base-washed polyethylene bottle and then adjusted with NaOH to achieve a pH of >11 and stoppered with a silicone stopper fitted with a strip of glass fiber filter to which 10 μl of 0.25 *N* H_2SO_4 has been spotted. The bottles are heated to 40°C and placed on a shaking table for 6 hr. The filters are then removed and dried for isotopic analysis. Recovery is ca. 65%, and the authors caution that other interferences may cause problems at low enrichment levels.

The fifth extraction method, specific for freshwaters, and unusable for brackish waters or saltwaters, is described as "simplicity itself" (Lipschultz, 1984). A measured quantity of zeolite molecular sieve (Union Carbide #W-85) is dried and placed onto a precombusted Whatman GF/F filter, and the freshwater sample is filtered. The sieve is then rinsed with deionized water several times and dried. Recovery of NH_4^+ is >95%, and there is a small background contamination. This method has been used in conjunction with distillation (Horrigan *et al.*, 1990b) as an alternative to evaporation and drying, to concentrate the NH_4^+ in the distillate.

Each of these techniques has advantages and disadvantages that must be weighed in working with different types of water. There are also several universal precautions that need underscoring. First, it is strongly preferred that recovery of the NH_4^+ be complete. Serious fractionation or discrimination of the isotopes can occur during distillation (reviewed by Hoefs, 1987) and during exchange reactions with zeolites (Bremner, 1965); because the rate of reaction or distillation is typically faster with the lighter isotope, this can lead to an overestimation of biological dilution. The extent of fractionation during chemical extraction or direct diffusion (e.g., Dudek *et al.*, 1986; Selmer and Sörensson, 1986; Kristiansen and Paasche, 1989) is currently unknown. Fractionation is not a problem if recovery is complete. Second, recovery of the NH_4^+ must be specific. Distillation methods that use excessively strong alkali will likely lead to contamination of the distillate with other labile nitrogenous compounds such as amino acids due to their degradation (Bremner, 1965). The extraction method of Dudek *et al.* (1986) has been shown to have little interference from organic nitrogen. Finally, it is essential that cross-contamination between samples be avoided. If there is 100% conversion of NH_4^+ for each and every sample, then virtually no cleaning of glass or plastic ware is necessary between samples, because no residue will remain. However, when recovery is <100%, cleaning with dilute HCl may be necessary. Alternatively, one may run the complete reaction in the glassware using distilled water between each sample to rid the walls of the glassware of any residual NH_4^+.

In all of the preceding methods, it is common practice to add a known quantity of unlabeled NH_4^+ ("carrier" nitrogen) prior to, or immediately after, distillation or extraction if the quantity of NH_4^+ in the water sample is insufficient to meet the mass requirements of the mass or emission spectrometer. The quantity of carrier can then be factored out in the final calculations of regeneration rates. We caution that the use of carrier nitrogen may "swamp" the overall signal; we describe the calculations and compromises with this approach more fully in Section IV.A.2.c.

b. NO_3^-, NO_2^- There are two general methods (Fig. 2). The first involves distillation of the sample, as already described, to remove NH_4^+; then DeVarda's alloy is added to reduce the NO_3^- and NO_2^- to NH_4^+, and the sample is redistilled. These methods are described in more detail in Chapters 2 and 7. The second involves the separation of NO_2^- from natural waters by the formation and subsequent extraction of an azo dye. This method was first described by Schell (1978) and has been improved by Lipschultz (1984).

c. Total dissolved organic nitrogen Total DON, or specific fractions thereof (e.g., amino acids, urea), is difficult to separate for ^{15}N. Molecular sieves and ion-exchange techniques have been described by Chan and Campbell (1978) and Schell (1974). A new method for the separation of DON from dissolved inorganic nitrogen (DIN) in marine samples, which permits the measurement of $DO^{15}N$ production during $DI^{15}N$ uptake and regeneration experiments, has been described by Bronk and Glibert (1991). Ion retardation resin (BioRad #142-7834; AG 11 A8, 50–100 mesh size) is used to separate $DI^{15}N$ from $DO^{15}N$, and the resulting $DO^{15}N$ is analyzed by mass spectrometry. When used in conjunction with ultrafiltration (e.g., Amicon #4305; 10,000-Dalton cutoff), low molecular weight $DO^{15}N$ can be separated from total $DO^{15}N$, and this distinction has been shown to be useful in determining what release processes may be occurring. An alternate method for separating $DO^{15}N$ from $DI^{15}N$ was described by Axler and Reuter (1986), but salt interferences restrict its applicability to freshwater systems. Note that regardless of the separation technique employed, these methods trace the release of DON that was derived from uptake processes; they do not trace all the possible pathways producing DON.

4. Preparation of Samples for Isotopic Analysis

All of the methods described previously share two common problems: (1) the conversion of the liquid sample to a form that can be introduced into a mass or emission spectrometer, and (2) the mass requirements of the mass or emission spectrometer must be met. The simplest approach is to evaporate the sample to near dryness and spot onto a precombusted glass fiber filter. Care must be taken to avoid atmospheric contamination during the evaporation process and after the filter has been dried. A second approach is the adsorption onto zeolite, as described earlier. Yet a third approach involves microdiffusion of the sample into a capillary tube

(Fiedler and Proksch, 1975). Once in this form, the samples are prepared and introduced into the instrumentation as described in Fiedler and Proksch (1975) and in Chapters 2 and 3 of this volume.

III. METHOD 2: SEDIMENT/WETLAND STUDIES

Nitrogen dynamics in sediments, particularly in shallow waters where benthic–pelagic coupling may be substantial, is the focus of much current interest, because nitrogen regeneration and efflux apparently supply a large portion of the biological nitrogen requirement of overlying waters. Similarly, bacterial production in the sediments can be substantial and may account for consumption of much of the detrital input.

To date, there has not been a great number of stable isotope studies in sediments; indeed, each study reported has contributed to methodological evolution and development. ^{15}N tracer studies of sediments have generally focused on the role of the sediment microbiota in NH_4^+ regeneration rather than on the concurrent assimilation of nitrogen (Blackburn, 1988). It is premature at this point to prescribe a standard procedure for sediment assays. While many of the provisos, cautions, and recommendations for bulk-water tracer analysis apply also to assessment of nitrogen uptake and regeneration in sediment systems, determination of biological transformations in sediments requires consideration of a number of unique and sometimes problematic aspects. We will focus on the unique factors that must be considered in taking an isotopic approach in sediment studies.

A. Measurement of Regeneration and Consumption

The NH_4^+ isotope dilution procedure was adapted from procedures applied in soil science (Kirkham and Bartholomew, 1955), first used in aquatic sediments by Blackburn (1979) and subsequently employed in several studies (Iizumi *et al.*, 1982; Blackburn and Henriksen, 1983; Bowden, 1984; Smith and DeLaune, 1985). While the emphasis in benthic studies has been in assessing the total rate of microbial NH_4^+ production, the methods, as in water column studies, also allow for a simultaneous estimate of NH_4^+ consumption. Operationally the method has generally been used as a measure of organic matter deamination, although several processes [e.g., deamination, NO_3^- reduction to NH_4^+, nitrogen fixation (Fig. 1)] can contribute to the production of NH_4^+ and account for dilution of a $^{15}N/^{14}N$ isotope ratio artificially increased over natural abundance levels. Assimilation has generally been considered as the primary route of consumption (particularly when incubations are performed anaerobically and thereby preclude nitrification; see later).

1. Incubation Protocols

Several factors need to be considered in establishing the most appropriate procedures for an experimental series (Capone, 1983). These include the recognition of relevant chemical gradients within the sediments (particularly O_2 and NH_4^+), enrichment levels with respect to ambient NH_4^+, and incubation time.

Relative to well-mixed waters, both predictable and apparently random variations in chemical constituents in sediments are of direct relevance in sample assay and treatment. For instance, O_2 concentrations often decrease and NH_4^+ levels increase downcore by >100-fold within centimeters of the sediment–water interface in coarse sands and within millimeters in finer-grained sediments. Predominating microbial processes also co-vary with downcore chemical gradients, as energetically favorable electron acceptors and labile organics are depleted (Capone and Kiene, 1988). This microbiological variation has direct implication for the extent of NH_4^+ regeneration. Furthermore, extreme variation in nitrogen pools and transformations can occur at the same horizon in adjacent sediment samples (e.g., Capone and Bautista, 1985).

Experimental protocols need to be developed with respect to the specific questions being addressed. Determination of *in situ* rates are probably most accurately measured by intact "in the core" procedures that attempt to preserve a sediment sample near its natural state with respect to chemical gradients (Jørgensen, 1978). In brief, such procedures isolate a segment of sediment in a mini core; this is typically an open-ended syringe barrel or glass tube sealed with gas-tight stoppers and with provisions for microsyringe injection ports (covered by septa or silicone sealant) along the barrel. Substrate is injected in small (microliter) volumes through the ports along the syringe barrel. After discrete incubation periods, cores are generally fractionated and the NH_4^+ pools within a particular horizon isolated for isotopic analysis.

Individual core incubations are subject to several limitations. Coring can potentially disrupt infauna and result in release of cellular material that may cause stimulation of microbial activities. Assay of individual intact cores generally requires large numbers of replicates to overcome natural variability (Bowden, 1984) or to establish time courses of activity because each core is destructively sampled. Without homogenization or shaking, the equilibration of added substrate in the core depends on diffusion and should be considered, particularly when assaying larger cores. Because a gradient of NH_4^+ concentration in the core is likely, it is difficult to achieve uniform enrichment by multiple injection within a core or among cores. Cores are typically sealed during the often-extended incubations to maintain anaerobic conditions. This ignores aerobic activity near the sediment–water interface.

While larger core sizes may mitigate the possible effect of infaunal disruption, diffusional problems would be aggravated. Assay of relatively thin core segments would compensate for the objection of traversing gradients but imposes greater sample manipulation and more tedious experimental design.

Alternately, sediments from a core or from a specific horizon within a core are mixed, often with a volume of seawater (i.e., slurried), combined with the isotope spike, dispensed into sample vials, and incubated (Blackburn, 1979; Bowden, 1984). Because the majority of sediment bacteria are tightly attached to sediment particles, gentle blending does not appear to disrupt these populations dramatically. On the one hand, mixing sediment ensures rapid distribution of the isotope and, with homogenization, allows more facile detection of differences among sampling sites or experimental treatments. Assays of batches of sediments simplifies experimental design and allows for time-course sampling (e.g., Bowden, 1984). On the other hand, any natural gradients of key constituents within the sediment are destroyed. There is the general sense that, for certain microbial activities and, in particular, competing, interdependent, or coupled reactions (e.g., nitrification–denitrification), destruction of the natural gradients and spatial relationships of the microbes compromises the ability to assess accurately *in situ* activities. Another approach may be the use of perfusion methods to infuse label into selectively undisturbed cores (Capone and Carpenter, 1982) or with *in situ* perfusion chambers. Such an approach has recently been applied in tropical seagrass systems (O'Donohue *et al.*, 1991).

Blackburn and Henriksen (1983) compared mixing with intact cores for determining regeneration rates by NH_4^+ isotope dilution. For anaerobically incubated coastal sediments, there was excellent agreement between the two procedures at stations with fine-grained sediments and at horizons down to 10 cm. At a sandy station, however, the mixing procedure yielded rates about threefold greater in several segments shallower than 6 cm. In contrast, Bowden (1984) noted in anaerobically incubated saltmarsh sediments over fourfold stimulation with mixing, which also dramatically reduced the variance among replicates, compared to intact cores.

In the limited number of studies to date, most workers have chosen to conduct incubations under anoxic conditions (Blackburn and Henriksen, 1983; Bowden, 1984). However, the results of Blackburn and Henriksen (1983) mentioned earlier are provocative in that differences noted in the upper segment of a sandy station could well be related to deeper penetration of O_2 at this station and the differences in the indigenous microbiota and quality of organic matter in oxic, compared to anoxic, diagenesis (O_2 data, however, were not presented).

The time of incubation is an important consideration, particularly when attempting to estimate *in situ* rates. Ideally, incubations should be kept as short as practicable. Maximum sensitivity, and the ability to keep incubations brief, will vary as a result of the isotope enrichment levels (see Section II.B.2), analytical precision, and intrinsic biological activity in the sample. Assay periods reported range from about 0.5 hr to 1 day (Iizumi *et al.*, 1982; Blackburn and Henriksen, 1983; Bowden, 1984).

2. Determining Tracer Addition

Concentrations of NH_4^+ in sediments are generally much higher than in overlying waters. It should therefore be a relatively simple matter, with a foreknowledge of the *in situ* concentrations, to increase the isotope ratio to a point useful for detecting isotope dilution, without a significant absolute increase in the concentration of NH_4^+. However, the capacity for NH_4^+ to be adsorbed onto inorganic particles (Mackin and Aller, 1984) and, in intact core assays, the gradient of NH_4^+ need to be considered.

Adsorption of rapidly exchangeable NH_4^+ can vary from insignificant to several times the interstitial pool, depending on sediment minerology (Mackin and Aller, 1984). Hence, in attempting to estimate the appropriate injection of tracer to achieve a given enrichment, it is useful to know adsorption capacity and porosity of the sample. Alternatively, initial enrichments can be determined from zero time controls, given sufficient time for diffusional (for injection cores) and exchange equilibria to be reached among NH_4^+ pools (Bowden, 1984). Unfortunately, there is little information concerning the kinetics of NH_4^+ exchange between interstitial, rapidly exchangeable, and, presumably, slowly exchangeable pools. Workers typically allow up to 2 hr for KCl to extract "exchangeable" NH_4^+.

For intact core assays, downcore gradients of NH_4^+ dictate that adjustments in injection volume or concentration need be made along each core if constant enrichment is to be achieved or, alternatively, that calculations account for a continuum of initial isotope ratios downcore.

3. Sample Harvesting

In general, sample harvesting in isotope dilution studies has involved either destructive sampling of individual flasks (Blackburn, 1979; Bowden, 1984), subsampling of batch incubations (Iizumi *et al.*, 1982), or segmentation of intact cores (Blackburn and Henriksen, 1983) with the sample or subsample placed immediately in a KCl solution for extraction of NH_4^+. Extracted samples have been either prepared for isotope analysis or stored frozen. The protocols for KCl extraction do not appear to be standardized because the ratio of sediment to KCl volume, normality of KCl, and length of extraction, vary among studies to date. T. H. Blackburn (personal communication) recommends as a rough guide 10 g sediment plus 10 ml 2.0 *M* KCl for 0.5 hr at 40°C. The efficiency of KCl extraction, or the potential for chemical isotope fractionation, does not appear to have been critically evaluated in studies of aquatic sediment.

4. Preparation of Sample for Isotopic Analysis

Various procedures have been used for preparation of samples for mass or emission spectrometry, and the methods parallel those used for water column samples.

B. Measurement of Uptake and Mobilization

The dynamics of NH_4^+ in sediments has also been examined by more "classical" tracer procedures; i.e., a label is introduced into a sample and after an incubation period the appearance of that label in other pools is evaluated.

For uptake studies, tracking the flow of NH_4^+ into particulate or dissolved nitrogen pools in sediments presents difficulties in addition to those mentioned for isotope dilution and comparable water column studies. The organic content of sediments, both dissolved and particulate, greatly exceeds that of the overlying waters. Much of the organic material in sediments is refractory; only a small fraction of the organic material is actively metabolizing microorganisms or metazoans. Rice (1982) concluded that much of the nitrogen in organic detritus is nonlabile humic nitrogen, and that microbial biomass contributes little to the nitrogen pool. Similarly, Mayer *et al.* (1986) found that protein was only a small fraction of total sediment nitrogen and living biomass a small fraction of this protein pool. Thus, in tracing the fate of NH_4^+ in a sediment sample, it is necessary to either observe an enrichment against a very large background of detrital nitrogen (Capone, 1988) or devise methods for separation of relevant components of the particulate pool. Careful controls need to be run to factor out any strong abiotic adsorption of NH_4^+, which might otherwise be included with organic nitrogen pools.

IV. GENERAL MODELS AND APPLICATIONS

A. Calculation of Atom %

1. Basic Models and Sample Calculations

Following the notation of Fiedler and Proksch (1975), the percentage of ^{15}N (atom%) in a sample is given by

$$\%\ ^{15}\text{N} = \frac{\text{amount of } ^{15}\text{N (by atoms or weight)}}{\text{total N (by atoms or weight)}}. \tag{1}$$

Using either mass or emission spectrometers, one obtains the abundance (in peak heights, volts, curve area, etc.) of $^{28}N_2$ ($^{14}N^{14}N$), $^{29}N_2$ ($^{15}N^{14}N$), and sometimes (depending on instrument sensitivity), $^{30}N_2$ ($^{15}N^{15}N$). If the mass 30 peak is not measured, the atom% is calculated according to:

$$\%\ ^{15}\text{N} = \frac{100R}{2 + R} \tag{2}$$

where R is (mass 29)/(mass 28). Thus, for example, if the 29 value (in volts) is 2.429 and the 28 value (in volts) is 240.2, the atom% would be 0.503. If mass 30 is also measured, the atom% is calculated according to

$$\%\ ^{15}\text{N} = \frac{(\text{mass } 30) + \frac{1}{2}(\text{mass } 29)}{(\text{mass } 30) + (\text{mass } 29) + (\text{mass } 28)}. \tag{3}$$

Note that in the numerator only, ½ of the $29N_2$ appears because it is only this fraction that is labeled with ^{15}N.

To calculate atom% excess, the natural isotopic abundance of ^{15}N (typically 0.365%, but actually determined for each instrument daily or more frequently) is subtracted from the values calculated above. For further discussion on the derivation of these formulae, the inherent assumptions, and alternative procedures for calculating atom%, the reader is referred to Chapters 2 and 3 of this volume, Fiedler and Proksch (1975), and Buckley *et al.* (1985).

2. Problems and Correction Factors

Not surprisingly, the calculation of atom% may not be as straightforward as indicated by the preceding equations. Three types of problems frequently occur.

*a. **Background nitrogen*** In virtually all samples, especially those collected on glass fiber filters, background nitrogen ("blank") contributes to the mass of N_2 and isotopically dilutes the label. Thus, to correct for this contamination, the contribution of 29 and 28 by the blank must be subtracted from the respective peaks. For reasons described later, it is often difficult (depending on the specific instrumentation) to obtain a direct measurement of the 29/28 ratio of the blank; thus, it may be assumed that it contains nitrogen at atom% normal ratios, and the contribution of the blank is subtracted in relation to the mass of a blank sample (e.g., a blank glass fiber filter). Thus, a blank-corrected R takes the following form:

$$R = \frac{(\text{mass } 29) - (B/S)(R^*)}{(\text{mass } 28) - (B/S)} \tag{4}$$

where (B/S) is the ratio of the total mass of the blank sample to the total mass of the experimental sample, and R^* is the (mass 29)/(mass 28) ratio of normal atmospheric nitrogen.

b. Samples of low total nitrogen content A commonly encountered situation is that the mass of sample being analyzed is low relative to the mass requirements of the instrument (specifically mass spectrometers). This is especially true for uptake studies conducted in oligotrophic waters, where several liters of water must often be filtered to obtain the minimum required biomass on the filter for analysis. The minimum sample size varies for different instruments, but, as a general rule, 1–2 μmol nitrogen is needed for most mass spectrometers, while samples as small as 0.3 μmol may be analyzed on most emission spectrometers. Depending on the configuration of the particular instrument being used, samples at the lower limit may show spuriously high R values due to fractionation at the inlet at low gas pressures. This effect has been recognized for over four decades (Honig, 1945). Fortunately, the physical behavior of the gas flow is predictable, and correction equations can be developed for each instrument. However, it is best to avoid samples of low nitrogen content when doing critical work.

c. Carrier corrections A third problem or correction factor, itself a solution to the problem of low mass, is the correction for "carrier" nitrogen (see Section II.B.3.a). In this case, the measured atom% of the analyzed sample will include the contribution of the carrier as follows:

$$\text{measured atom\%} = \frac{(Ms)(\text{atom\% } S) + (Mc)(\text{atom\% } C)}{(Ms + Mc)} \tag{5}$$

where Ms is the mass (concentration) of nitrogen in the sample, Mc is the mass of carrier nitrogen, atom% S is the atom% of the sample without carrier (i.e., the unknown), and atom% C is the atom% of the carrier nitrogen (i.e., atom% normal). The equation can be rearranged to solve for atom% S as follows:

$$\text{atom\% } S = \frac{(\text{measured atom\%})(Ms + Mc) - Mc(\text{atom\% } C)}{Ms} \tag{6}$$

It is strongly suggested that, when carrier is added, the minimum amount of carrier is used to simply satisfy the mass requirements of the mass or emission spectrometer. Due to the fact that the concentration of carrier is large relative to the concentration of the sample (otherwise no carrier would be needed), and due to the fact that it appears in Eq. (5) twice (once in the numerator and once in the denominator), small errors in this estimate can lead to errors in the calculation of atom% of the sample.

B. Calculation of Uptake

1. Basic Models

The specific uptake rate, or velocity (V), is calculated from the equations derived by Dugdale and Goering (1967) and Sheppard (1962). They are

$$V = \frac{\text{atom\% excess}}{\text{atom\% enrichment} \times \text{incubation duration}} \tag{7}$$

where atom% excess is calculated as described in the preceding section, and atom% enrichment is calculated according to

$$\text{atom\% enrichment} = \frac{\text{total } ^{15}\text{N added}}{\text{total } ^{15}\text{N added} + \text{total ambient N substrate}} \tag{8}$$

Thus, V represents the rate of nitrogen transport from one compartment (i.e., seawater) to another compartment (i.e., phytoplankton). The dimensions are amount of N taken up $(\text{time})^{-1}$ divided by the amount of N in the phytoplankton, which reduces to $(\text{time})^{-1}$. To calculate an absolute rate of uptake or transport (ρ), the specific uptake rate is multiplied by the particulate nitrogen (PN) content of the sample:

$$\rho = V \times \text{PN} \tag{9}$$

making sure that the following cautions are heeded.

2. Problems and Corrections Factors

a. PN In practice, the PN content of a sample is determined on a separate aliquot of the sample using a carbon-hydrogen-nitrogen (CHN) analyzer or determination of the pressure of the resulting N_2 gas in the inlet line of the mass spectrometer, if appropriately calibrated. A major disadvantage, however, in using the pressure in the inlet line for the calculation of PN is that if the mass of nitrogen in the combined nitrogen is so large as to be out of the range of the pressure sensor, one typically bleeds off some gas, and an accurate determination of the PN is then impossible to obtain. The advantage of the former is that the analysis tends to have higher precision; the advantage of the latter is that a separate sample need not be taken.

Additionally, the correct determination of ρ uses the value of PN determined at the end of the experiment rather than at the beginning of the incubation (Collos, 1987). Although with short incubations, the error attributable to the use of the initial value rather than the final PN value is small, with longer incubations, significant under- or overestimates of the uptake rate can result if the PN increases or decreases over the incubation interval.

Natural samples also contain varying amounts of nitrogen detritus, the proportion of which cannot easily be determined. Thus, the resulting uptake rate for the living fraction of material will be underestimated (Dugdale and Goering, 1967). However, by comparing the C : N ratio and the PN : chl ratio, one can gain some insight into the detrital contribution. Elevations in both ratios indicate a higher percentage of detritus and/or, in the latter case, heterotrophic organisms. In sediments, much of this detrital nitrogen may be nonlabile humic nitrogen (Rice, 1982).

b. Isotope dilution During incubations with natural samples, the atom% of the aqueous pool becomes progressively diluted with time. We recognize and take advantage of this with measurements of regeneration. It is important that isotope dilution be recognized in the determination of uptake as well. Several models have been presented in the literature for the correct calculation of time-varying (progressively "diluted") atom% (Glibert *et al.*, 1982; Laws, 1984; Glibert and Garside, 1989). These have been discussed in detail by Glibert (1988). Briefly, the denominator of Eq. (7) must be modified to reflect an exponentially decreasing atom%. Without such modification, uptake can be seriously underestimated [division in Eq. (7) would be by an erroneously large denominator].

c. Uptake of other nitrogen compounds In nature, several nitrogen sources may be present and simultaneously utilized by the primary producers. When only one of those compounds is investigated with the use of ^{15}N-labeled substrates, concurrent uptake of the other compounds may contribute to mass signal 28, which may cause significant underestimates of the uptake rate (Collos, 1987; Lund, 1987). Mathematical treatments to calculate uptake when simultaneous uptake of several nitrogen sources occurs are given by Collos (1987) for the situation where the final PN is known and by Lund (1987) where only the initial PN value is known.

d. Discrimination Isotope discrimination during the process of nitrogen uptake has long been recognized, and this is described in more detail in Chapter 4. Isotopic discrimination is usually not considered in isotopic tracer studies, however, because it is usually thought that the mass spectrometers (and even more so, emission spectrometers) used in tracer studies are not sensitive enough to detect very small changes. Yet, there are experimental conditions under which discrimination may lead to measurable changes in the atom% of the dissolved nitrogen pool. For example, during uptake, the lighter isotope is preferentially utilized, and, with time, the dissolved nitrogen remaining will become progressively enriched, if little or no simultaneous isotope dilution due to regeneration is occurring. The extent to which the effect may be observed will depend on the rate of uptake, the initial enrichment, and the sensitivity and precision of the instrument. In this situation, the uptake rate would be overestimated if Eq. (7) were applied without correcting for the increasing atom% of the NH_4^+, but this effect is very small. In contrast, when regeneration occurs, as is typically the case in natural samples, the effect of isotope discrimination during uptake may be ignored.

Isotope discrimination can also occur during the chemical analysis of samples for isotope enrichment. For example, as discussed earlier, there can be fractionation during distillation of NH_4^+ (Hoefs, 1987), during which the lighter isotope distills faster than the heavier isotope. If there is incomplete recovery of the nitrogen, the amount recovered will be isotopically lighter than the nitrogen in the original sample. Again we caution investigators that distillation or extraction procedures must yield complete recovery to avoid this problem (see also Chapter 2).

C. Calculation of Regeneration and Mobilization

1. Basic Models

The basic models for the calculation of regeneration in sediments and water column samples were simultaneously put forth by Blackburn (1979) and Caperon *et al.* (1979) and have been recently reviewed by Glibert (1988) and Glibert and Garside (1989). Knowing the change in substrate concentration, and the change in ^{15}N atom% in the dissolved pool, the rates are solved for as follows:

$$P_{(t)} = P_0 + (d - u)t \tag{10}$$

and

$$\ln\,[R_{(t)} - 15n] = \ln\,(R_0 - 15n) - [d/(d - u)][\ln P_{(t)}/P_0] \tag{11}$$

where $P_{(t)}$ and P_0 are the ambient concentrations of NH_4^+ at the end and beginning of the incubation period (t), $15n$ is the normal atmospheric atom% of ^{15}N (~0.365%), u is the absolute uptake rate, and d is the regeneration (dilution) rate. Note that although the convention in the literature has been to identify the atom% at time 0 and time t as R_0 and $R_{(t)}$, this R is not equal to the R in Eq. (2)–(4), which represents the ratio of (mass 29)/(mass 28). The dimensions of u and d in this model are (mass N)(vol^{-1})(time^{-1}).

Modifications to this basic model have been presented by Glibert *et al.* (1982), Garside and Glibert (1984), and Laws (1985). The Garside and Glibert model applies the same basic equations above in a forward-difference computer model. The Laws model is an analytic solution to, again, these same basic equations. Which one to use is a matter of investigator choice, because, although there has been considerable debate in the literature as to the best approach, all will yield identical values when the parameters uptake and regeneration are computed (Garside and Glibert, 1984; Laws, 1985; Glibert and Garside, 1989). Differences among models only arise when other less commonly used parameters, such as "total incorporation" (Laws, 1985), are compared. Glibert *et al.* (1982) discuss the relative merits of calculating uptake according to Eq. (9) (modified to account for isotope dilution) or Eqs. (10) and (11). Equations (10) and (11) depend on the mass balance of the NH_4^+ pool sizes, which at low concentrations of NH_4^+ is extremely imprecise.

The calculation of urea regeneration, when the dual isotope technique described earlier is used, takes basically the same form as Eqs. (10) and (11). We assume that changes in the specific activity of ^{14}C-urea represent the same changes that simultaneously occur in ^{15}N atom%. The enrichment (or specific activity) of ^{14}C-urea (E) is calculated as:

$$E = A/[F * P] \qquad (12)$$

where A is the activity (dpm) in the experimental sample, F is the volume of the sample that was counted, and P is the concentration of urea. The fractional change in enrichment (FC) with time is calculated as

$$\mathrm{FC} = (E_0 - E_{(t)})/E_0 \qquad (13)$$

where E_0 and $E_{(t)}$ represent the enrichment at time 0 and time final, respectively. This value is then used to calculated the ^{15}N atom% ($R_{(t)}$) according to

$$R_{(t)} - 15n = (R_0 - 15n)(1 - \mathrm{FC}) \qquad (14)$$

where R_0 is determined according to

$$R_0 = [S/P_0 + [(P_0 - S)(15n/(100),/P_0]](100) \qquad (15)$$

where S is the concentration of added ^{15}N-urea, and P_0 is the total (added plus ambient) concentration of urea substrate at time 0. These values are then substituted in Eq. (11) to obtain the regeneration rate. The procedure for calculating uptake is identical to that described earlier for ^{15}N isotope studies. Additional details are provided in Hansell and Goering (1989), noting corrections to their Eqs. (5), (7), and (8); our corresponding Eqs. (12), (14), and (15) should be used instead.

Lastly, the calculations take yet a different form when production of a different ^{15}N-labeled compound is monitored relative to the initial ^{15}N tracer addition (Fig. 1). These calculations are more analogous to those for uptake, except that the product pool is not PN but, rather, the dissolved substrate of interest (Horrigan *et al.*, 1990a; Bronk and Glibert, 1991). Thus, to calculate the rate of flux from dissolved NH_4^+ to dissolved NO_3^- (mindful that only the net rate is being calculated and that intermediate steps are involved), one would use the following equation:

$$\rho = \frac{(\text{atom\% excess in } NO_3^-)(NO_3^- \text{ concentration})}{(\text{atom\% enrichment in } NH_4^+)(\text{incubation duration})} \qquad (16)$$

where both values in the numerator are determined at the end of the incubation period.

2. Problems

a. Mass balance of isotope Are uptake and regeneration described fully and accurately by the basic preceding models? The answer is a resounding maybe. As noted above, Glibert *et al.* (1982) emphasized that the value u in Eqs. (10) and (11) does not necessarily equal the value ρ determined from the incorporation of ^{15}N label in the particulate material [Eq. (9)], even when corrected for isotope dilution as noted earlier (Section IV.B.2.b). This inequality represents either loss of ^{15}N once taken up by phytoplankton, to release products in another nitrogen form, such as NO_2^- or DON, or incomplete recovery of the plankton (especially the bacterial fraction) on the filters (Glibert *et al.*, 1982, 1985; Laws, 1984; LaRoche and Harrison, 1987). Alternatively, it may represent adsorption of label to sediment or container walls. Glibert *et al.* (1991) present an example of the amount of ^{15}N recovered during time-course studies conducted during two seasons in the outflow plume of the Chesapeake Bay estuary. During the winter, the recovery was incomplete, and other evidence suggests that ^{15}N-labeled DON may have been released by physiologically stressed phytoplankton. However, during the summer, there was virtually complete recovery of the added ^{15}N in the subsequently measured particulate and dissolved fractions. Although it is desirable to obtain a complete inventory of ^{15}N label for all time points during an incubation experiment, it is extremely difficult because the analytical burden is substantially increased to account for the mobility of label to all other nitrogen pools. However, such studies involving the major pools and transformation processes have been undertaken in the Delaware River (Lipschultz *et al.*, 1986) and in the Southern California Bight (Ward *et al.*, 1989).

b. Apparent increases in atom% with time It has occasionally been observed that, during the course of tracer incubation experiments, the atom% of the substrate pool becomes enriched with time. Enrichment of the substrate pool appears to occur simultaneously with substrate depletion. Increases in atom% obviously imply that regeneration is negligible relative to the rate of uptake. Increases may be due to isotope discrimination in the uptake step (see Section IV.B.2.d), or adsorption and subsequent desorption, whereby the lighter isotope is favored, leaving behind an NH_4^+ pool that becomes smaller but "heavier." Lastly, and most simply, it may be due to problems associated with some step in the analysis of atom% (see particularly discussion on physical behavior of gases in Section IV.A.2.b and "carrier" corrections in Section IV.A.2.c). Should this phenomenon be observed in the course of experimentation, the investigator should be aware that analytical problems are likely but that biological discrimination in the absence of regeneration could contribute to this observation.

V. CONCLUSIONS

In this review, we have attempted to address the major methodological problems and approaches in using ^{15}N tracer techniques in water column and sediment studies. At this time, it is impossible to outline a cookbook method suitable for all circumstances. The appropriate approach to answer one set of questions may be entirely wrong if a different set of questions are being addressed. There have been many criticisms raised recently regarding ^{15}N methods—some going so far as to question whether or not these methods should continue to be used. We do not agree that the problems are impossible to overcome. Well-designed and executed experiments will continue to lead to new insights into the pathways and rates of nitrogen transformations in aquatic and sediment systems.

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10

^{13}N *Techniques*

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I. INTRODUCTION

Four isotopes of nitrogen with atomic masses of 12, 13, 16, and 17 have the probability of an unstable, transient existence. The ^{13}N isotope is the longest-lived of these; it disintegrates with a half-life of 9.96 min (effectively 10 min) by emission of a positron (β^+, 1.2 MeV maximum emission energy) giving rise to ^{13}C. As a positron loses its kinetic energy, it pairs with an electron in the medium and in a subsequent annihilation reaction forms two gamma photons each of 0.511 MeV energy traveling in nearly opposite directions. The gamma radiation is detectable outside of the human body; thus, ^{13}N and related short-lived positron-emitting radionuclides have their most prominent application as radiopharmaceuticals in positron emission tomography in diagnostic nuclear medicine (Raichle, 1981; Vaalburg and Paans, 1983).

There are also advantages in the use of ^{13}N in environmental and basic biological research owing to its high specific activity and sensitivity of detection in the absence of any natural abundance (for units and terms, see Section II). The specific activity of ^{13}N alone is 1.88×10^{10} Ci/mol. Assuming that at least 0.05 nCi can be measured in the decay of ^{13}N, it is theoretically possible to detect $2\text{–}3 \times 10^{-21}$ mol of the isotope (Tiedje *et al.*, 1979). Based on experimentally generated specific activities of 10^6 Ci/mol of $^{13}NH_4^+$ (Parks and Krohn, 1978) or 10^8 Ci/mol of $^{13}NO_3^-$ (Hollocher *et al.*, 1980), it is reasonable to predict routine detection of 5×10^{-17} to 5×10^{-19} mol. This is in comparison to reports of

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5–30 × 10^{-8} mol of ^{15}N at 99 atom% excess (Matthews and Hayes, 1978) and 10^{-12} mol of ^{14}C (Tiedje *et al.*, 1979). These advantages of ^{13}N have best been exploited in basic biological studies examining the immediate products of nitrogen assimilation and dissimilation at environmental and physiological concentrations of the substrates.

The major limitation of ^{13}N in biological studies is its 10-min half-life; it is not amenable to transport and storage, and experiments must be done within the constraints of the half-life. Analysis of metabolic products of ^{13}N transformations have been done from 15 min to 4 hr after end of bombardment. Thus, the use of ^{13}N is restricted to institutions operating a suitable particle accelerator for generation of the radionuclide. However, a considerable number of institutions worldwide maintain Van de Graaff generators and medical cyclotrons of low-acceleration energies (relative to high-energy physics instruments) (Helus and Wolber, 1983).

The history of ^{13}N application in biological studies has been detailed in two excellent reviews in the past decade (Krohn and Mathis, 1981; Cooper *et al.*, 1985). Specific examples of basic biological and medical uses also are provided in the volume edited by Root and Krohn (1981). Although ^{13}N experiments are technically difficult to conduct, the citations in the preceding reviews illustrate the unique and unequivocal results that can be obtained. The goal of this chapter is to present current methods for ^{13}N production, manipulation and analysis after biological incubations.

II. UNITS OF RADIOACTIVE DECAY AND MEASUREMENT OF ^{13}N

A. Units and Specific Activity

The curie (Ci) has been the historical standard unit of radioactive decay. It is defined as the disintegration of 3.7 × 10^{10} atoms of radioactive nuclei per second, with the standard source being 1 g of ^{226}Radium. The standard international (SI) unit is the becquerel (Bq), defined as one disintegration per second. The relationships are

$$1\ \mathrm{Bq} = 2.703 \times 10^{-11}\ \mathrm{Ci} \quad \text{and} \quad 1\ \mathrm{Ci} = 3.7 = 10^{10}\ \mathrm{Bq}$$

The specific activity per mol (g-atom) of a pure radionuclide can be defined by the decay equation:

$$-dn/dt = \lambda N$$

where λ is the decay constant or ln 2/half-life in seconds, N is the number of radioactive nuclei, at 1 mol, *N* is Avogradro's number (6.02 × 10^{23} nuclei/mol). Thus, for pure ^{13}N at $t_{1/2}$ = 10 min, the maximum mol sp. act. is (0.693/600 sec)(6.02 × 10^{23}/mol) or 6.95 × 10^{20} Bq/mol, or at 3.7 × 10^{10} Bq/Ci is 1.88 × 10^{10} Ci/mol.

B. Measurement of ^{13}N

Because ^{13}N decays by positron emission followed by annihilation to gamma photons, its activity has been monitored using a variety of commercially available ionization gauges, gas flow proportional counters, and solid and liquid scintillation systems (for a thorough discussion of detection systems, see Knoll, 1979).

Solid-fluor scintillation spectroscopy using thallium-activated sodium iodide crystals [NaI(Tl)] in a variety of shapes and sizes coupled to photomultiplier tubes is the basis of most of the currently available gamma photon counting systems (Bicron Corporation, Newbury, Ohio; EG & G Ortex, Oakridge, Tennessee; Harshaw Chemical Company, Solon, Ohio; Packard Instruments Company, Inc., Downers Grove, Illinois). The NaI(Tl) crystal may be drilled to form a well counter, the geometry of which gives maximal counting efficiency. Such well counters, or single NaI(Tl) crystals, also function adequately at a distance from the source under conditions of 2pi geometry. Alternatively, a pair of NaI(Tl) crystals can be positioned directly opposite each other facing the source and connected by coincidence counting circuitry (within 100 nsec; Tiedje *et al.*, 1979). The coincidence counting utilizes the antiparallel movement of the gamma photons from the site of annihilation and allows for detection of low amounts of radiolabeled metabolic products when analyzed under conditions of high background, such as in the additional presence of ^{13}N targets during multiple experimental runs. The NaI(Tl) systems can be calibrated using ^{22}Na as the standard source (β^+, $t_{1/2}$ = 2.62 yr, annihilation gamma emission energy of 0.511 MeV). A calibrated NaI(Tl) well counter is frequently used to establish the "true" activity of a ^{13}N sample, which can then be used to standardize other detection systems (Tiedje *et al.*, 1979).

Ionization gauges, because of their portability, are convenient for routine monitoring of β particles and gamma photons from the irradiated target and generated $[^{13}N]N_2$ at the site of biological incubation (Wolk *et al.*, 1976; J. C. Meeks, unpublished). The commercial instruments, as health physics survey meters (e.g., Victoreen Instruments Company, Clevelend, Ohio; Technical Associates, Canoga Park, California), record units of radiation exposure as roentgen per hour (R/hr). The value of R/hr can be converted to mCi by the following equation:

$$\mathrm{R/hr} = (I_g)(\mathrm{mCi})/d^2,$$

where d is the distance from the source in centimeters, and I_g is the dose rate per hour at 1 cm, or 5.885 for ^{13}N (Wolk *et al.*, 1974).

The positron can be detected as ionizing radiation by gas flow proportional detectors, which are essentially insensitive to the gamma photons. Most proportional detectors can be fabricated in-house. Commercial examples are the detectors incorporated into radiochromatogram scanners (e.g., Model 7200 series, Packard Instruments Company, Downers Grove, Illinois). These proportional counters operate with 2pi geometry in a hemispherical chamber with a collimator and a thin anode wire under high voltage (1.1 kV) with a continuous flow of counting gas (e.g., 4% isobutane–96% helium). In the absence of a standard ^{13}N solution, the counters can be roughly calibrated using ^{32}P (β^-, maximum emission energy of 1.7 MeV).

The positron emission can also be monitored by liquid scintillation spectroscopy using organic fluors. The activity is most conveniently measured in a ^{32}P energy window and the instrument calibrated with ^{32}P. There is no apparent activation of the organic fluors by the gamma photons. However, Glass *et al.* (1985) observed that ^{13}N decay results in Cerenkov light and that the photomultiplier tubes in liquid scintillation systems function as Cerenkov detectors. The Cerenkov emission was best detected in glass scintillation vials in the absence of any liquid and in a low-energy tritium window.

C. Decay correction

The radioactivity measured at any time must be corrected for decay to standard reference time. The reference time can arbitrarily be set to any point near the beginning of the experiment; the end of bombardment is the obvious choice in consideration of yield, but the end of chemical conversion to the particular molecular substrate is equally valid in biological experiments. The exponential form of the decay equation can be used directly in correction:

$$C_t = C_o \cdot e^{-\lambda t}$$

where C_t is the radioactive counts (per second or minute) at the time of counting, C_o is the counts corrected to the reference time, λ is the decay constant or $\ln_2$/half-life (seconds or minutes), and t is $t - to$, or the elapsed time (seconds or minutes). Using time in minutes, this equation reduces and rearranges to

$$\text{cpm at reference time } (C_o) = (C_t)2^{t-to/10}$$

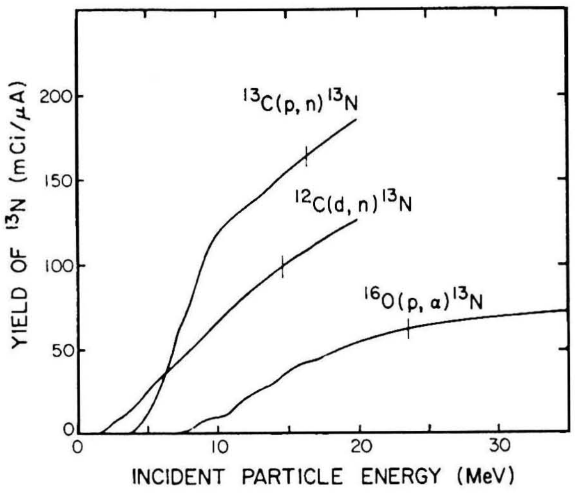


Figure 1 Production yields of ^{13}N by three nuclear reactions. The yield in mCi per μA beam current was calculated as a function of particle acceleration energy. [Reproduced with permission from Austin, S. M., Galonsky, A., Bortins, J., and Wolk, C. P. (1975). A batch process for the production of ^{13}N-labeled nitrogen gas. *Nuclear Instr. Method.* **126**, 373–379.]

III. GENERATION OF ^{13}N—NUCLEAR REACTIONS AND PRODUCTS

Among the eight accelerator reactions that have been used for the production of ^{13}N (Vaalburg and Paans, 1983; Cooper *et al.*, 1985), three are the most common; $^{12}C(d,n)^{13}N$, $^{13}C(p,n)^{13}N$, and $^{16}O(p,\alpha)^{13}N$. Yield and activation characteristics of these reactions are given in Fig. 1. The choice of substrate and reaction depends on the specific properties of the accelerator and the most convenient target design. Because a collaborative arrangement between the biological researcher and nuclear physicists and/or the accelerator support staff is the only practical approach to designing the complete target system, explicit details will not be given here. Examples of some target systems are given by Helus and Wolber (1983).

A. $^{12}C(d,n)^{13}N$

The deuteron-induced neutron-replacement reaction on ^{12}C has a theoretical yield of up to 100 mCi/μA of beam current at particle energies of 15 MeV (Fig. 1). Experimental yields of 60 mCi of ^{13}N trapped after 10 min of irradiation with 7.5 MeV deuterons at 15 μA current have been reported

(Tilbury *et al.*, 1971). The 0.33-MeV threshold particle energy required to initiate the conversion allows for yields of up to 200 μCi of ^{13}N with a Van de Graaff generator accelerating deuterons at 2.5 MeV and 1 μA of beam current (McNaughton and More, 1979). The low threshold also minimizes the production of other radionuclides with half-lives >1 sec. The target systems have utilized molecular substrates as solids, such as Li_2CO_3 (McNaughton and More, 1979) or graphite (Buckingham and Clark, 1972), or gases, such as CO_2 (Jones *et al.*, 1977) or CH_4 (Tilbury *et al.*, 1971; Straatmann, 1977).

The primary ^{13}N molecular product depends on the reaction of the radicle ^{13}N atom with the molecular substrate. For example, the Li_2CO_3 substrate yields about 85% radiochemically pure $^{13}NO_3^-$ with 3% $^{13}NO_2^-$; the remainder has not been identified. The ^{13}N-nitrogen oxides are recovered by first dissolving the target product in distilled water containing Amberlite 252 H^+ ion-exchange resin to remove Li followed by gentle heating to remove CO_2 (McNaughton and More, 1979). The product of irradiating both the graphite (Buckingham and Clark, 1972) and CO_2 (Jones *et al.*, 1977) substrates is $[^{13}N]N_2$, presumably as the diatomic molecule. The CH_4 substrate yields primarily $^{13}NH_3$ (with <5% $C_xH_x{}^{13}NH_2$) that is trapped in dilute acid (Tilbury *et al.*, 1971).

B. $^{13}C(p,n)^{13}N$

The proton bombardment of ^{13}C has the highest theoretical yield of ^{13}N per μA of beam current (Fig. 1). Experimental yields of 100 mCi of ^{13}N following 20 min of irradiation with 11 MeV protons at 1 μA are routine (Austin *et al.*, 1975; J. C. Meeks and C. P. Wolk, unpublished results). The accelerating particle threshold energy of the reaction is also within the range of Van de Graaff generators, especially those of tandem design (Nickles *et al.*, 1978). However, this reaction has been used extensively only with the low-energy accelerator at Michigan State University (for a review, see Meeks, 1981), which was replaced with a heavy ion accelerator in 1979. Details of the pneumatic shuttle target cell are in Austin *et al.* (1975) and Tiedje *et al.* (1979). The substrate in that target system was 18.6 mg of amorphous carbon, 97 atom% ^{13}C (Monsanto Research Corporation, Mound Laboratory, Miamisburg, Ohio). The ^{13}N hot atom product remains trapped in the carbon crystalline lattice from which it can be processed by Dumas combustion into $[^{13}N]N_2$ (Austin *et al.*, 1975) or by acid digestion into $^{13}NH_4^+$ (Thomas *et al.*, 1977).

C. $^{16}O(p,\alpha)^{13}N$

The proton irradiation of ^{16}O and resulting α-particle displacement has the highest threshold energy of the three reactions at about 9 MeV and gives the lowest theoretical yield per μA of beam current (Fig. 1). Nevertheless, experimental yields of 1 Ci of ^{13}N with water as substrate and 20 MeV protons at 40 μA current have been reported (Parks and Krohn, 1978), although yields of 0.5 Ci are more typical with 15 μA (Vaalburg *et al.*, 1975) or 20 μA (Chasko and Thayer, 1981) beam currents. The primary product of the $^{16}O(p,\alpha)^{13}N$ reaction with water as substrate is $^{13}NO_3^-$, ranging from 90 to 99% radiochemical purity, with $^{13}NO_2^-$ constituting 3–10% and $^{13}NH_4^+$ 0–2%. The exact proportions of the three molecular species, however, appears to vary depending, perhaps, on trace impurities or dissolved gases in the target water and the proportions must be determined in each laboratory; the variability is discussed by Krohn and Mathis (1981) and the hot atom (recoil) chemistry by Tilbury (1981). The natural abundance of ^{18}O in oxygen-containing substrates also results in variable contamination with ^{18}F (β^+, $t_{1/2}$ = 110 min) as a consequence of the low threshold (2.5 MeV) of the $^{18}O(p,n)^{18}F$ reaction. Moreover, at acceleration energies greater than 16.6 MeV, the $^{16}O(p,pn)^{15}O$ reaction occurs yielding substantial ^{15}O (β^+, $t_{1/2}$ = 122 sec), which can introduce significant measurement error if the reaction products are used immediately after the end of bombardment.

Irrespective of the preceding limitations, the $^{16}O(p,\alpha)^{13}N$ reaction appears to be the current target system of choice in many European (Vaalburg and Paans, 1983) and U.S. (Cooper *et al.*, 1985) cyclotron-based laboratories. The advantages of this reaction include the following.

1. The use of distilled and deionized water as substrate in static or recirculating target cells. In some low-volume (e.g., 1 ml) static targets, the total yield of ^{13}N is understandably low (3–16 mCi; Tiedje *et al.*, 1979), but it is economical to use ^{18}O-depleted water as substrate in such target cells and avoid the generation of ^{18}F (Skokut *et al.*, 1978). Even a low initial presence of ^{18}F will introduce considerable counting error during routine analysis of biological reactions by 1.5–2 hr after end of bombardment. The larger volume recirculating target systems have the added advantages of high total yield (0.5–1.0 Ci) and increased cooling of the target substrate during bombardment, thus avoiding cavitation in the target cell.
2. The ease in remote manipulation of the irradiated product because of the variety of valves, pumps, and switches that are now available for liquid handling (Chasko and Thayer, 1981).
3. Versatility in the potential to synthesize four of the molecular species of inorganic nitrogen that are important in biology and predominant in the environment (i.e., ^{13}N-labeled NO_3^-, NO_2^-, NH_4^+, and N_2).

The 60-ml recirculating water target system in place at the University

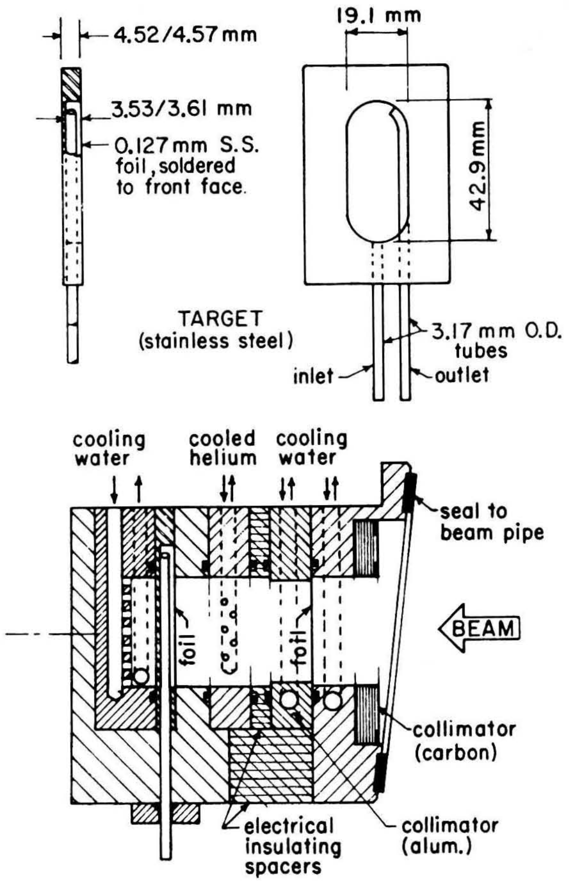


Figure 2 Schematic of the stainless steel target (upper) and assembled target block (lower) used at UCD/CNL in the production of ^{13}N using water as substrate in the $^{16}O(p,\alpha)^{13}N$ reaction. [Reproduced, with permission, from Parks, N. J., and Krohn, K. A. (1978). The synthesis of ^{13}N labeled ammonia, dinitrogen, nitrite, and nitrate using a single cyclotron target system. *Int. J. Appl. Radiat. Isot.* **29**, 754–756.]

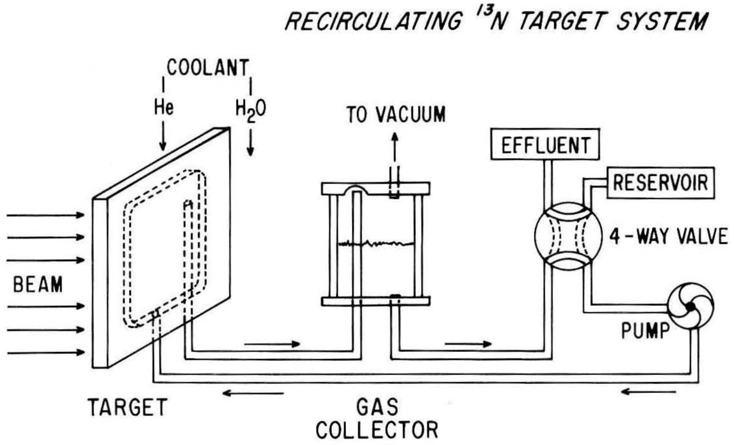


Figure 3 Schematic of the recirculating water target system in place at UCD/CNL. [Reproduced, with permission, from Parks, N. J., and Krohn, K. A. (1978). The synthesis of ^{13}N labeled ammonia, dinitrogen, nitrite, and nitrate using a single cyclotron target system. *Int. J. Appl. Radiat. Isot.* **29**, 754–756.]

of California, Davis, Crocker Nuclear Laboratory (UCD/CNL) will serve as a paradigm in application of the preceding advantages. Details of the target block are given in Fig. 2, and a schematic of the system is in Fig. 3 (Parks and Krohn, 1978; Parks, 1982). The magnetically coupled, variable-speed centrifugal pump (Model 12A-41-316, Micropump Corporation, Concord, California), selector valve, and gas collector/sight glass are located in a lead brick-shielded area just outside of the main wall of the cyclotron vault and are connected to the target block by 1.5 mm ID nylon tubing. The volume of the entire system is approximately 60 ml and the water is circulated at a rate of 60–100 ml/min. The four-way selector valve allows simultaneous replacement of target water as it is removed. The target water is transferred by the recirculation pump through a 2- × -5-cm aluminum column packed with alumina/MnO$_2$ that removes contaminating ^{18}F (Hollocher *et al.*, 1980) and is collected in 60-ml reservoirs in a lead brick-shielded work station. The target water is then either used directly in the synthesis of $^{13}NO_2^-$ or $^{13}NH_4^+$ (Section IV) or, in a protocol developed by Chasko and Thayer (1981), the ^{13}N-anions are concentrated using high-performance liquid chromatography (HPLC) and minicolumns packed with a high-performance anion exchange material (e.g., Partisil-10 SAX, Whatman, Inc.). A schematic of the concentration and associated purification system is shown in Fig. 4. The radioanions are eluted from the minicolumn using physiological saline (0.9% NaCl, w/v) or 30 m*M* phosphate buffer, pH 3.0, and used to synthesize $^{13}NH_4^+$ (Section III), used directly in biological experiments, or $^{13}NO_2^-$ and $^{13}NO_3^-$ individually purified by passage through an analytical HPLC anion-exchange column. The advantages of the HPLC processing include concentration of the radioanions (e.g., up to 230 mCi $^{13}NO_3^-$/ml), purification from other ions (Fig. 5), and ultimate suspension in the physiological solution of choice.

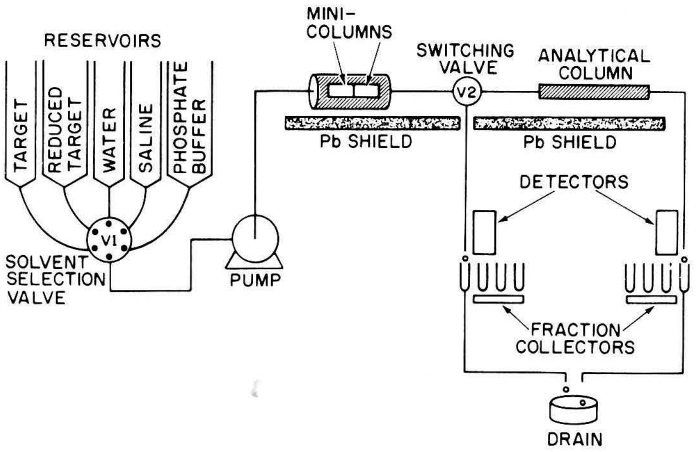


Figure 4 Schematic of the system used for concentration and purification of $^{13}NO_3^-$ and $^{13}NO_2^-$ from the irradiated water target at UCD/CNL. [Reproduced, with permission, from Chasko, J. H., and Thayer, J. R. (1981). Rapid concentration and purification of ^{13}N-labelled anions on a high performance anion exchanger. *Int. J. Appl. Radiat. Isot.* 32, 645–649.]

IV. SYNTHESIS OF ^{13}N MOLECULAR SPECIES

A. $^{13}NO_3^-$

^{13}N-nitrate is the primary product of two of the nuclear reactions discussed earlier. The total yield of radioactive product in the $^{12}C(d,n)^{13}N$ reaction with Van de Graaff accelerators, however, is in the low microcurie range (McNaughton and More, 1979). Conversely, the total yield of $^{13}NO_3^-$ in the $^{16}O(p,\alpha)^{13}N$ reaction is routinely around 0.5 Ci (Vaalburg *et al.*, 1975; Parks and Krohn, 1978; Chasko and Thayer, 1981). The $^{13}NO_3^-$ has been purified from any contaminating $^{13}NO_2^-$ and $^{13}NH_4^+$ by two methods. Skokut *et al.* (1978) (modified by Meeks, 1981) used a 2-min incubation of small (1–5 ml) volumes of target water with 1% (v/v) H_2O_2 at pH 2.0 (addition of 0.05 ml of 0.01 *M* formic acid) to oxidize $^{13}NO_2^-$ to $^{13}NO_3^-$, after which the solution is vacuum-evaporated to dryness to remove H_2O_2 and HCOOH; the residue is suspended in 0.01 *M* NaOH and vacuum-evaporated to dryness a second time to remove $^{13}NH_3$. The residue from the final evaporation is suspended in the desired volume of H_2O or buffer. Catalase (50 μg) is added to remove any residual H_2O_2. The oxidation and evaporations take about 10 min.

In the second approach, Chasko and Thayer (1981) used preparative and analytical HPLC on anion-exchange columns to remove $^{13}NH_4^+$ and separate $^{13}NO_3^-$ and $^{13}NO_2^-$. A schematic of the system used at UCD/CNL is shown in Fig. 4. The radioanions in the target water bind to the exchange resin in the preparative minicolumn (guard columns packed in the laboratory with Partisil-10 SAX, or purchased as cartridges; SAX-GU, Brownlee Labs, Santa Clara, California) and any trapped $^{13}NH_4^+$ is removed by additional (2 min at 10 ml/min) flushing with distilled water. The radioanions are eluted from the minicolumns with 30 m*M* phosphate buffer, pH 3.0. The minicolumns are then flushed for 1–2 min with saline (0.9% NaCl; 6 ml/min) to convert the exchange material to the Cl^- form, which will then allow for binding of NO_3^-/NO_2^- in the next production run (neither NO_3^- nor NO_2^- will displace PO_4^{2-} from the material). In the absence of analytical chromatography, the $^{13}NO_2^-$ is oxidized to $^{13}NO_3^-$

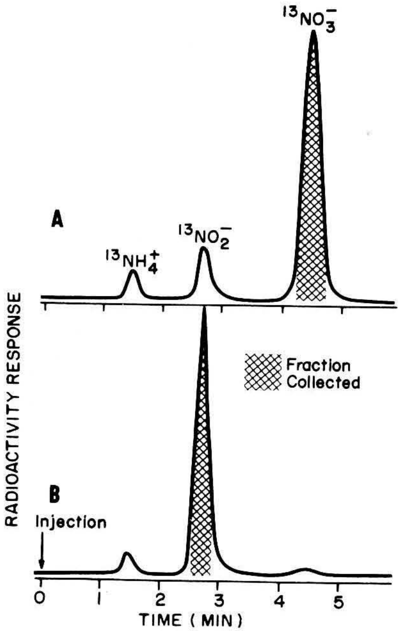


Figure 5 (A) HPLC radiochromatogram of the target solution following irradiation of 60 ml of recirculating water with 20-MeV protons at 20 μA for 20 min at UCD/CNL. The radioions were separated by analytical HPLC on a Partisil-10 SAX column, eluted with 30 m*M* phosphate, pH 3.0, at 3 ml/min. More than 90% of the target product is $^{13}NO_3^-$. (B) HPLC radiochromatogram of target water passed through a copperized cadmium reduction column before separation on a Partisil-10 SAX analytical column under the same conditions as above. In the production system, the analytical HPLC column is replaced with minicolumns, which do not resolve $^{13}NO_3^-$ and $^{13}NO_2^-$. [Reproduced, with permission, from Chasko, J. H., and Thayer, J. R. (1981). Rapid concentration and purification of ^{13}N-labelled anions on a high performance anion exchanger. *Int. J. Appl. Radiat. Isot.* 32, 645–649.]

with 3% (v/v) H_2O_2, neutralized, and treated with catalase to remove any remaining peroxide. The concentration and chemical purification steps take about 12 min to complete. The resulting solution is 99% radiochemically pure $^{13}NO_3^-$ and typically contains 140 mCi in 0.6 ml (Chasko and Thayer, 1981), with a specific activity of approximately 5×10^6 Ci/mol (Krohn and Mathis, 1981). Chasko and Thayer (1981) discuss a number of practical considerations in the HPLC concentration protocol; foremost is the observation that the anion-exchange media appear to be sensitive to intense radiation and need to be replaced after processing 5–10 Ci of target solution.

For ultrapure (>99%) preparation of either radioanion (Fig. 5), the target solution or minicolumn concentrate can be loaded onto an analytical SAX column, eluted with 30 m*M* phosphate, pH 3.0, and the peak fractions (<0.5 ml) of $^{13}NO_2^-$ (shorter retention time) or $^{13}NO_3^-$ collected with a fraction collector; the fractions are identified by an audible pocket dosimeter or rate meter. The analytical column takes about 10 min to complete. In our experience, the purity gained through the analytical separation is rarely necessary.

B. $^{13}NO_2^-$

^{13}N-nitrite is a variable, but generally minor, product of the $^{16}O(p,\alpha)^{13}N$ reaction with water as substrate. McElfresh *et al.* (1979) adapted a copperized cadmium reduction column protocol for a large volume water target. Approximately 20 g of cadmium filings are treated in sequence with 2 *N* HCl, 0.3 *N* HNO_3, and 2 *N* HCl. The acid-treated filings are kept in solution with minimal exposure to air and washed twice for 20 min with 0.08 *M* $CuSO_4$. The copper precipitate is removed by distilled water flushing and the filings stored in a solution of 0.15 *M* NH_4Cl at pH 5.2. The copperized cadmium filings are packed into a column of variable size, depending on the volume of target water to be processed (Pasteur pipette to 7 mm × 25 cm). The column ends are plugged with acid washed (2 *N* HCl) copper wool and the column filled with a solution of 0.15 *M* NH_4Cl; the filings are then poured into the column avoiding, as much as possible, exposing the filings to air. The column is stored under 0.15 *M* NH_4Cl and washed with distilled water before use. The target water is transferred to the column and recirculated using a peristaltic pump. One pass through the column is generally sufficient to convert >99% of the $^{13}NO_3^-$ to $^{13}NO_2^-$; multiple passes (from 6 to >20) result in a linear decline in $^{13}NO_2^-$ paralleled by an increase in $^{13}NH_4^+$ (McElfresh *et al.*, 1979). McElfresh *et al.* (1979) transferred the eluent (40–60 ml) from the reduction column to a rotatory evaporation apparatus (Brinkman Instruments Inc., Westbury, New York) containing 0.15 ml of 0.1 *M* NaOH to remove contaminating $^{13}NH_4^+$ and concentrate the $^{13}NO_2^-$. The entire procedure takes about 15 min and yields about 3 ml of $^{13}NO_2^-$ at 30 mCi/ml.

Alternatively, the $^{13}NO_2^-$ in the reduction column eluent can be purified by anion-exchange HPLC with the minicolumns used in concentration of target radioanions. This method as it is used at UCD/CNL takes less time and extraneous equipment because the HPLC system is in operation. Chasko and Thayer (1981) used a HPLC pump to transfer the eluent from a reduction column to anion-exchange minicolumns and recovered 90 mCi of $^{13}NO_2^-$ in 0.6 ml. Tiedje *et al.* (1981) adapted the preceding protocol to a small volume water target using columns the size of Pasteur pipettes and gravity flow rather than a peristaltic pump.

C. $^{13}NH_4^+$

^{13}N-ammonia is a direct product of the $^{12}C(d,n)^{13}N$ reaction using CH_4 as substrate in a continuous flow target system. (Tilbury *et al.*, 1971). The total yield was reported as 60 mCi of ^{13}N in 7 ml of saline solution, which is sufficient for most biological experiments. The product, however, also contains about 5% organic-[^{13}N]amines plus carrier NH_4^+.

Thomas *et al.* (1977) synthesized $^{13}NH_4^+$ from the product of the $^{13}C(p,n)^{13}N$ reaction by acid digestion. The irradiated amorphous carbon target is digested in a 100-ml Kjeldahl flask in the presence of 0.4 g potassium dichromate, 0.2 g potassium sulfate, and 2 ml of concentrated sulfuric acid. The acid digestion is for 10 min starting at 100°C and increasing to 250°C. At the end of digestion, the suspension is neutralized by addition of 5 ml of saturated sodium borate (pH 10) and 8 ml of 40% (w/v) NaOH, and 2 ml of saturated silver sulfate is added to reduce the nitrogen oxides to NH_4^+. The $^{13}NH_4^+$ is recovered by gentle vacuum distillation and trapped by freezing in liquid N_2; the distillation is monitored with an ionization gauge until there is no further increase in activity in the trap. The yield is typically 6 mCi in 1.0–1.5 ml with a specific activity of 2×10^4 Ci/mol (Thomas *et al.*, 1977).

The currently preferred method of $^{13}NH_4^+$ synthesis is by reduction of the $^{13}NO_3^-/^{13}NO_2^-$ products of the $^{16}O(p,\alpha)^{13}N$ reaction. The reduction of NO_3^-/NO_2^- is catalyzed by either titanous chloride (Krizek *et al.*, 1973) or granular Devarda's alloy (50% copper–45% aluminum–5% zinc) (Vaalburg *et al.*, 1975).

The titanous chloride method was automated by Ido and Iwata (1981). In this protocol, target water is pumped into a sealed reaction flask containing 4 ml of saturated NaOH, and then 4 ml of a 10% (w/v) solution of $TiCl_3$ is pumped from a syringe into the reaction flask to initiate the reduction. The flask is heated for about 8 min with halogen lamps until the solution reaches about 150°C, which is reported optimal for vacuum-distilled recovery of $^{13}NH_4^+$. Additional syringe pumps are used in sequence to rinse the reaction flask and add NaOH for the next reduction–distillation. Absolute yields of $^{13}NH_4^+$ were not given by Ido and Iwata (1981), but time corrected yields of 87–91% of the initial radioactivity were noted and a 99.9% radiochemical purity was claimed. The advantage of $TiCl_3$ over Devarda's alloy as a catalyst is the ease in remote addition of a solution rather than a solid; its major disadvantage is the handling of a reactive pyrophoric powder.

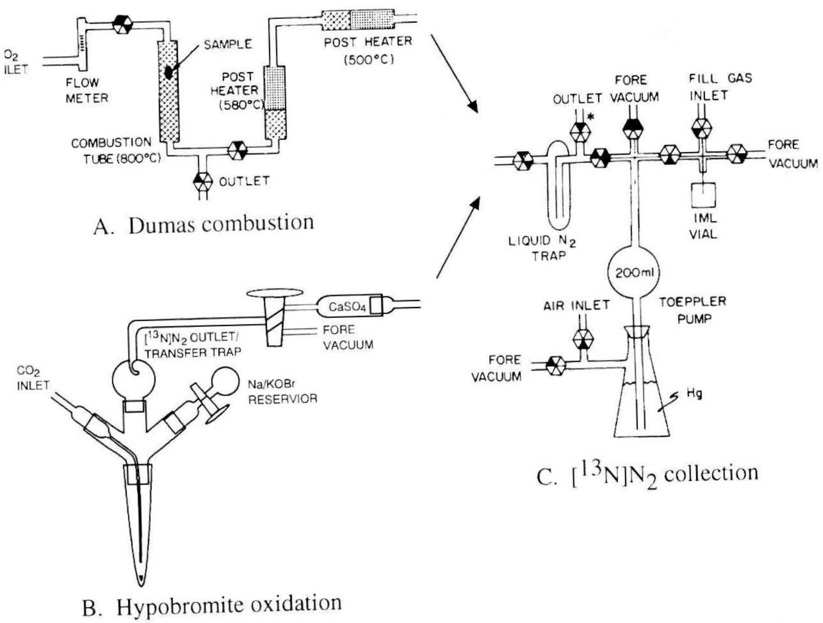
Vaalburg *et al.* (1975) reported more reproducible yields of $^{13}NH_4^+$ using Devarda's alloy compared to $TiCl_3$ to reduce $^{13}NO_3^-/^{13}NO_2^-$. As a partial consequence of that observation, and the ease in assembling routine glassware for the synthesis system, Devarda's alloy has had the most widespread application in synthesis of $^{13}NH_4^+$ from water targets. In the method developed by Vaalburg *et al.* (1975), 10 ml of irradiated target water is introduced into a reaction flask containing 0.2 g of Devarda's alloy, 5 ml of saturated NaOH, and 0.1 ml of ethanol. The $^{13}NH_3$ is removed by 2–3 min of steam distillation and recovered in 6–8 ml of condensate. The ethanol was included to moderate the exothermic reaction. Vaalburg *et al.* (1975) reported yields of approximately 0.5 Ci of $^{13}NH_4^+$ from target water irradiated with 19 MeV protons at 15 μA beam current. Variations of the reaction employing different quantities of target water, Devarda's alloy, and NaOH were subsequently adapted by others (see Krohn and Mathis, 1981; Vaalburg and Paans, 1983; Cooper *et al.*, 1985). The reagents used for the reaction contain various amounts of ammonium; thus, the distilled product contains between 63 and 100 μM NH_4^+ (Meeks, *et al.*, 1983, 1985b; Cooper *et al.*, 1985) and the specific activity is consequently lower than theoretical. From literature surveys, Cooper *et al.* (1985) reported specific $^{13}NH_4^+$ activities that range from 1 to 5 × 10^5 Ci/mol. Using steam distillation, Parks and Krohn (1978) determined a specific activity of 10^6 Ci/mol, whereas we have used vacuum distillation and recovered $^{13}NH_4^+$ at up to 7.9 × 10^5 Ci/mol (Meeks *et al.*, 1983, 1985b).

Parks and Krohn (1978) present a detailed schematic of an apparatus designed for Devarda's alloy-catalyzed reduction, steam distillation, and condensate collection of the $^{13}NH_4^+$. A more simple version using Bantamware (Kontes, Vineland, New Jersey) is currently used at UCD/CNL. Target water (45 ml) or HPLC minicolumn concentrate (3 ml) is pumped in through one neck of a 250-ml round-bottom three-neck flask containing 10–15 ml of saturated NaOH and a magnetic stir bar and sitting in a heating mantle on top of a magnetic stirrer; the system is prewarmed to about 65°C. The reaction is initiated by adding 1 g Devarda's alloy from a 1-dram vial connected to one neck of the flask by flexible rubber tubing. The reaction flask is connected to a 12-ml graduated distillation receiver via a distilling trap, Liebig condenser, vacuum adapter, and appropriate angled adapters. The distillation receiver contains 2 ml of acidic buffer (5 m*M* Mops or Mes, pH not adjusted, also containing 7 m*M* formic acid) or 0.35 *M* formic acid (for subsequent generation of $[^{13}N]N_2$). A slight vacuum is pulled on the reaction flask through the trapping solution; too vigorous an application of vacuum results in rather violent transfer of aerosols containing NaOH. The major peak of activity is distilled between 2.5 and 4.5 min after addition of Devarda's alloy. While 80% of the total can be recovered by 6.5 min and 94% by 10 min of distillation, some of the activity is trapped in the system and does not reach the receiver. Thus, in a total volume of 2.5–3.0 ml, typical total real time activities average 62 mCi, ranging from 27 to 130 mCi, with a specific activity of 1.5–7.9 × 10^5 Ci/mol (Meeks *et al.*, 1983, 1985b). The $^{13}NH_4^+$ solution is transferred from the distillation receiver to a second receiver–transfer vessel using a hand-operated vacuum pump (Nalgene).

D. $[^{13}N]N_2$

^{13}N-dinitrogen can be obtained as a direct product of the $^{12}C(d,n)^{13}N$ reaction using graphite as substrate. The graphite target was described by Buckingham and Clark (1972; see also Clark and Buckingham, 1975). The critical step is to sweep the irradiated target with CO_2; the CO_2 effectively erodes the graphite matrix and releases the $[^{13}N]N_2$. Any CO formed during the erosion is oxidized to CO_2 by flow of the target product through a copper oxide furnace and the CO_2 removed by absorption in a 2 *N* NaOH trap. The $[^{13}N]N_2$ is ultimately solubilized in physiological saline. The procedure yields about 1–3 mCi of $[^{13}N]N_2$ in a 10-ml volume, with unknown specific activity. A similar graphite target system was recently used by Caldwell *et al.* (1984), except in this case the ^{13}N is recovered by spontaneous exchange with infiltrated $^{14}N_2$. The gas phase is removed from the target with a syringe and yields 10–20 μCi of $[^{13}N]N_2$ in 7–15 ml, also with unknown, but presumably low, specific activity.

A similar N exchange reaction has been exploited by Suzuki and Iwata (1977) and Parks and Krohn (1978) in the $^{16}O(p,\alpha)^{13}N$ reaction using 0.1 *M* aqueous NH_3 as substrate. Attachment of a spirometer to the gas collection vessel (Fig. 3) allows for collection of continuously produced ^{13}N gaseous products. Using this continuous system, 200 mCi of ^{13}N can be collected, of which about 75% is $[^{13}N]N_2$, with the remainder primarily ^{13}N-nitrogen oxides. In addition to the impurities, a disadvantage of this protocol, and that of the previously mentioned graphite target, is the low specific activity of 67–80 Ci/mol (Suzuki and Iwata, 1977; Parks and Krohn, 1978). Nevertheless, these low activities have been used to obtain biologically and medically useful information.



A. Dumas combustion

B. Hypobromite oxidation

C. $[^{13}N]N_2$ collection

Figure 6 Schematics of the systems used for low-volume generation and processing of high specific activity $[^{13}N]N_2$. (A) Dumas combustion system for the solid product of the $^{13}C(p,n)^{13}N$ reaction using a semiautomated nitrogen analyzer. (B) Hypobromite oxidation of $^{13}NH_4^+$ in liquid solution. The $^{13}NH_4^+$ is synthesized by Devarda's alloy reduction of the products of the $^{16}O(p,\alpha)^{13}N$ reaction with water as substrate. (C) $[^{13}N]N_2$ collection and transfer system. The $[^{13}N]N_2$ is swept from either generation system by a stream of CO_2 and enters the collection system via the LN trap, which freezes the carrier CO_2 and any oxides of N. The Toeppler pump is used to compress and transfer the $[^{13}N]N_2$ to the reaction or storage vial. [Panels A and C are reproduced, with permission, from Austin, S. M., Galonsky, A., Bortins, J., and Wolk, C. P. (1975). A batch process for the production of ^{13}N-labeled nitrogen gas. *Nuclear Instr. Method.* **126**, 373–379.]

A semiautomated process for synthesis of $[^{13}N]N_2$ at very high specific activity (1×10^4 Ci/mol) in a low volume (<0.1 ml) was developed specifically for experiments in biological N_2-fixation (Austin *et al.*, 1975). The protocol utilizes Dumas combustion of the product of the $^{13}C(p,n)^{13}N$ reaction. The system consists of two basic components: (1) a commercial combustion apparatus (Model 29 Nitrogen Analyzer, Coleman Instruments Corporation, Maywood, Illinois) for conversion of ^{13}N trapped in the lattice of the carbon substrate and carrier $^{14}NO_3^-$ to diatomic $[^{13}N]N_2$ (Fig. 6A), and (2) a spiral liquid nitrogen (LN) trap to freeze the CO_2 sweep gas coupled to a Toeppler mercury pump to collect and transfer the $[^{13}N]N_2$ (Fig. 6C). The irradiated target is added to a combustion tube that is filled approximately ⅔ with cuprox (CuO) and contains 1.8 g cuprox fines plus 0.18 mg carrier KNO_3 on top of the cuprox; the tube is then filled with additional cuprox and the radioactivity determined with an ionization gauge. The filled combustion tube is inserted in the analyzer and purged with ultrapure CO_2 (Coleman Grade, Matheson, Newark, California) at 300 ml/min for about 3 min. The sample is then subjected to combustion in a CO_2 atmosphere at 800°C for about 6 min in the automatic cycle of the instrument. At the end of combustion, the products in the tube are swept with CO_2 at 30 ml/min through two sequential post columns, both containing a section of cuprox (40% of the total volume) and a separate section of cuprin (Cu) (60% of the total volume), at 580°C and 500°C to reduce nitrogen oxides to $[^{13}N]N_2$. The carrier CO_2 and any remaining nitrogen oxides are trapped by freezing in the LN trap. The $[^{13}N]N_2$ is collected by expansion into an evacuated 200–300-ml reservoir on top of the Toeppler pump. The $[^{13}N]N_2$ is then compressed into a reaction or transfer vial by opening the bottom reservoir of the Toeppler pump to air. For incubation, the vial is filled to atmospheric pressure with Argon/CO_2 (99/1, v/v). The direction of radioactive gas flow is controlled with appropriate stainless steel 1-mm bore stopcocks (Circle Seal Controls, Anaheim, California). This entire generation process takes about 13 min from the time of filling the combustion tube to compression of the radioactive gas into the reaction vial. Over a 2-yr period based on 190 $[^{13}N]N_2$ generations, an average of 12.03 mCi was delivered to the reaction vials with yields of 10.2% real time and 26% time corrected (J. C. Meeks and C. P. Wolk, unpublished results). The combustion process has been used to generate $[^{13}N]N_2$ only with the solid product of the $^{13}C(p,n)^{13}N$ reaction; the yields are about 10-fold lower using the HPLC-concentrated products of the $^{16}O(p,\alpha)^{13}N$ reaction with H_2O as substrate (J. C. Meeks, unpublished results).

$[^{13}N]N_2$ can also be prepared by oxidation of the $^{13}NH_4^+$ produced by $TiCl_3$ or Devarda's alloy-catalyzed reduction of the products of the currently preferred $^{16}O(p,\alpha)^{13}N$ reaction with H_2O as substrate (see Vaalburg and Paans, 1983). The oxidation is generally catalyzed by alkaline hypobromite (Vaalburg *et al.*, 1981) in a manner similar to preparation of $^{15}NH_4^+$ for mass spectrometric analysis (Burris, 1972); it can also be catalyzed by hypochlorite, but this reaction has not been examined in any detail (N. J. Parks, unpublished results). Vaalburg *et al.* (1981) adapted the hypobromite protocol to ^{13}N. Their stock hypobromite solution was prepared by adding 3 ml Br_2 to 100 ml ice cold 8% (w/v) NaOH (stable for 1 in the dark at 4°C). The $[^{13}N]N_2$ is generated by adding 0.2 ml of stock NaOBr to 2.0 ml $^{13}NH_4^+$ solution in a 3.0-ml sealed vial. After 10 min for reaction, 0.1 ml of a 20% (w/v) ascorbic acid solution is added to destroy excess NaOBr and the solution neutralized by addition of 0.2 ml of 0.5 *M* phosphate buffer, pH 7.4. From a 10-ml target solution irradiated with 20-MeV protons at 1 μA beam current, the protocol yields about 10 mCi of $[^{13}N]N_2$ dissolved in 2.5 ml of liquid, at undefined specific activity.

The alkaline hypobromite oxidation of $^{13}NH_4^+$ has also been used to produce gaseous $[^{13}N]N_2$ at high specific activity (3.9×10^4 Ci/mol); the $^{13}NH_4^+$ is derived from the $^{16}O(p,\alpha)^{13}N$ reaction products using Devarda's alloy (Meeks *et al.*, 1985a). In this protocol used at UCD/CNL, the oxidation step replaces the combustion step in the two-component generation–collection system developed by Austin *et al.* (1975) (Fig. 6B). A stock Na/KBr solution is made by slowly dissolving 25 g solid Br_2 (or 8 ml liquid Br_2), 25 g NaBr, and 1 g KI in 200 ml distilled water. The stock solution is stable for >1 yr when stored in a glass stoppered bottle at room temperature in the dark. Alkaline hypobromite is prepared the day of use by combining 1 volume of stock Na/KBr, 1 volume of saturated NaOH, and 3 volumes of distilled H_2O; subsamples of 3 ml are transferred to custommade 6-ml reservoirs (Fig. 6B), vacuum-degassed and stored under ultrapure CO_2. The generated $^{13}NH_3$ is trapped in 2 ml of 0.35 *M* formic acid, also containing 1.5 μmol NH_4Cl as carrier. The $^{13}NH_4^+$ solution, in a 12-ml centrifuge tube (14/20 standard taper) with a triangular magnetic stir bar, is placed in the rack of a magnetic stirring apparatus (Model MS-7 with MT-72 Table, Tri-R Instruments, Inc. Rockville Centre, New York). A custommade head piece (Fig. 6B; 14/20 standard taper) holding a CO_2 sparging line, the gas exit line, and a port for the evacuated Na/KOBr reservoir is inserted and the solution is sparged for 1 min with ultrapure CO_2 at 30 ml/min. After CO_2 sparging, the Na/KOBr reservoir is attached and the solution is vacuum-degassed for 2 min. Alkaline hypobromite is added to initiate the oxidation by opening the stopcock to the evacuated reservoir. After 30 sec of reaction, the $[^{13}N]N_2$ is swept from the solution by sparging for 30–60 sec with CO_2 at a flow rate of 1–5 ml/min. Water vapor in the gas stream is removed with a 2-×-10-cm column of anhydrous $CaSO_4$ (Drierite) and carrier CO_2 plus any nitrogen oxides or hypobromite frozen in the LN trap. The $[^{13}N]N_2$ is collected and transferred using the Toeppler pump as described in the Dumas combustion procedure. The entire process from beam off, through concentration of target water and sequential generation of $^{13}NH_4^+$ and $[^{13}N]N_2$, to start of biological incubation takes about 20 min. On average of 72 oxidations over a 2-yr period, delivery of 35.96 mCi of $[^{13}N]N_2$ to the 1–3-ml reaction vials was obtained, reflecting a 58% time-corrected yield of $[^{13}N]N_2$ from $^{13}NH_4^+$ (J. C. Meeks, unpublished results).

V. BIOLOGICAL INCUBATIONS AND ANALYSIS OF METABOLIC PRODUCTS

A. Intracellular Accumulation

The translocation and distribution of ^{13}N-labeled NO_3^-, NH_4^+, or N_2 from the point of entry in plant tissues can be monitored by placement of multiple Gieger-Mueller tubes along the plant axis (Caldwell *et al.*, 1984), by autoradiography on X-ray film between blocks of dry ice for 20–30 min (Deane-Drummond and Thayer, 1986), or by hand-sectioning of the tissue and scintillation counting. Accumulation of ^{13}N-labeled ions by microorganisms requires separation of the cells from the incubation medium; both direct filtration and density centrifugation can be used.

Betlach *et al.* (1981) examined time and concentration-dependent $^{13}NO_3^-$ uptake by *Pseudomonas fluorescens* by vacuum filtering 1-ml subsamples of cells through cellulose acetate filter pads (Metricel GA-6, Gelman Instrument Company). The filter pads are washed with 2–5 volumes of buffer or growth medium and radioactivity on the pads is quantitated by scintillation spectroscopy. Filtration and washing generally take 10–15 sec. A variation of this standard technique is to lyse cells filtered onto polycarbonate pads (Nuclepore) by incubation in the filter holder for 5 min with 5 ml cold 5–10% (w/v) trichloroacetic acid (vacuum off), followed by rinsing the precipitated cell debris three times with 5% cold trichloroacetic acid. The radioactivity on the filter pad is then determined to assess metabolism of the transported ions (Silver and Perry, 1981; Zehr *et al.*, 1988). Betlach *et al.* (1981), however, noted nonspecific binding of both $^{13}NO_3^-$ and contaminating ^{18}F to the various types of filter pads; thus, corrections must be made for such background by filtering killed or no cells.

To avoid possible loss of transported $^{13}NO_3^-$ by excretion during filtration, Thayer and Huffaker (1982) utilized centrifugation through silicone oils. A 0.2–0.5-ml mixture of Dow-Corning silicone oils (75% 550 and 25% 510-[50 CS], Thayer and Huffaker, 1982; 40% 550 and 60% dinonylphthalate, Scott and Nicholls, 1980) are added to 1.5-ml microcentrifuge tubes. The cell suspension (0.5 ml) is gently layered on top of the silicone oil, the tube is placed in a microcentrifuge, and at timed intervals 0.1–0.2 ml of stock ^{13}N-ion is added with a micropipette. At an appropriate time after the last sample is inoculated, all samples are centrifuged at maximum speed for 25 sec (most microcentrifuges take 5 sec to reach speed and 20 sec to stop). The incubation medium is removed by gentle pipetting for radioactive counting if desired and the silicone oils removed by aspiration. The cells in the pellet can be lysed by suspension in 80% (v/v) methanol and either counted directly or analyzed for ^{13}N-labeled metabolites. As an alternative, the microcentrifuge tubes may contain a 0.2-ml pad of 15% perchloric acid under the silicone oils to immediately lyse the cells after which the lysate can be analyzed (Scott and Nicholls, 1980).

B. Gaseous Products

The biological conversion of $^{13}NO_3^-$ by denitrification to the gaseous products ^{13}NO, [^{13}N]N_2O and [^{13}N]N_2 can be studied by gas stripping (Gersberg *et al.*, 1976; Tiedje *et al.*, 1979) or analytical gas chromatography (Tiedje *et al.*, 1979) of the products. In the gas stripping system, helium gas at 90 or 300 ml/min is flushed through a reaction flask containing magnetically stirred anaerobic cell or soil slurries and substrate $^{13}NO_3^-$ plus reduced carbon. The dominant gaseous products, [^{13}N]N_2O and [^{13}N]N_2, are swept from the suspension and trapped at LN temperature by freezing (N_2O + NO) or adsorption (N_2) in molecular sieve 13X (Linde Division, Union Carbide). A moisture trap of anhydrous $CaSO_4$ is placed between the incubation flask and LN trap(s) to prevent ice plugging of the gas lines. Gersberg *et al.* (1976) trapped the gases in the same vessel and monitored the sum of their radioactivity with a NaI(Tl) well counter. Tiedje *et al.* (1979) modified the system to separately trap [^{13}N]N_2O and [^{13}N]N_2 (Fig. 7). In this system, carrier and radioactive gases exit the incubation flask at 90 ml/min via 3.2-mm-OD silicone tubing through the $CaSO_4$ trap to a first LN trap of 3.2-mm OD aluminum tubing in a 5.2-cm-diameter flat coil to freeze out [^{13}N]N_2O plus any ^{13}NO. The remaining gasses pass to a molecular sieve 13X trap made by packing the matrix into a 6.4-mm-OD copper tubing in a coil of 6.5 cm in diameter; the trap is immersed in LN. Both traps are monitored with 7.6- × -7.6-cm NaI(Tl) crystals in a constant geometry.

An alternative to the gas stripping system is to adapt a standard gas chromatograph (GC) to a radioactive detector. Some advantages of GC over gas stripping are the potential to separate and detect NO, to verify the identity of all gaseous denitrification products, and to determine specific activity . A version of the system designed by Tiedje *et al.* (1979) is shown in Fig. 8. The GC uses a 1.8-m long × 3.2-mm OD stainless steel column packed with Porapak Q (Alltech Associates, Inc., Deerfield, Illinois); it operates at 50°C in the column oven, with helium at 30 ml/min as carrier gas and 0.5–1.0-ml injection volumes. Under the operating parameters, N_2 and NO coelute within 35 sec from the matrix, while N_2O is retained for about 82 sec. Two approaches can be taken to resolve the N_2 and NO. In the initial version of the system, a 1.8-m long × 3.2-mm-OD stainless-steel column of molecular sieve 5A is located downstream of the Porapak Q column. Gas flow through the molecular sieve column is open during injection and closed 35 sec after injection to isolate N_2 and NO; at about 165 sec after injection gas flow is redirected through the molecular sieve column to elute N_2 and NO in sequence (205 and 260 sec, respectively).

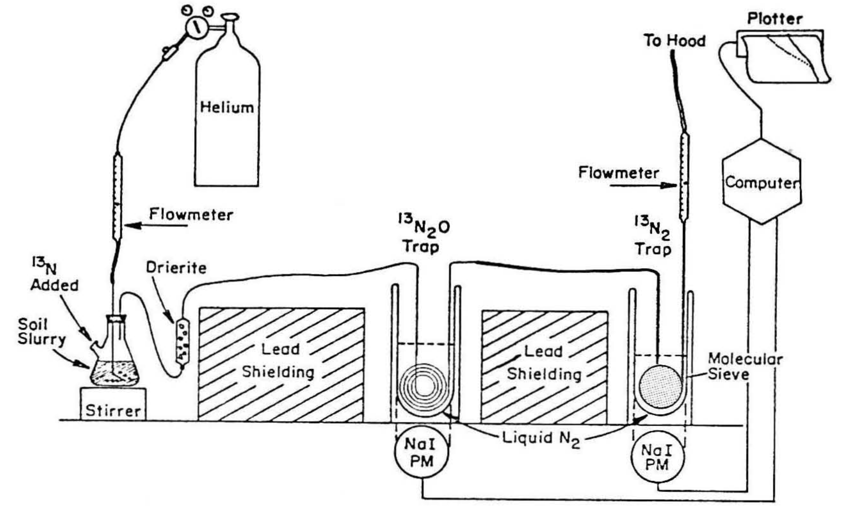


Figure 7 Schematic of the gas stripping system used to study denitrification. Gaseous products of $^{13}NO_3^-$/$^{13}NO_2^-$ reduction are swept from the reaction vial with helium at 90 ml/min and pass through a first LN trap to freeze ^{13}NO and [^{13}N]N_2O and then a molecular sieve 13X trap in LN to retain [^{13}N]N_2. The traps are monitored by NaI(Tl)-based scintillation detectors. [Reproduced from Tiedje *et al.* (1979) *Soil Science Society of America Journal,* Volume 43, No. 4, 1979, pages 709–716, by permission of the Soil Science Society of America, Inc.]

The second approach adopted by Sørensen *et al.* (1980) utilizes the differential freezing points of N_2O and NO compared to N_2. A 50-cm long × 0.32-cm-OD stainless steel loop is installed between the injection port and the Porapak Q column and immersed in LN. After the [^{13}N]N_2 has eluted, the loop is removed from LN and the warmed ^{13}NO and [^{13}N]N_2O gases are separated on the Porapak Q column. In both systems, the effluent from the columns passes through a microthermister detector to quantitate the total nitrogen products and then through a gas flow porportional counter with 4pi geometry to monitor radioactivity. The proportional counter operates at near 100% efficiency (Tiedje *et al.*, 1979).

C. Ionic Products

HPLC is the current method of choice to separate and quantitate $^{13}NH_4^+$, $^{13}NO_3^-$, and $^{13}NO_2^-$. The basic protocol was introduced by Tilbury and Dahl (1979) and has been adapted to various production and

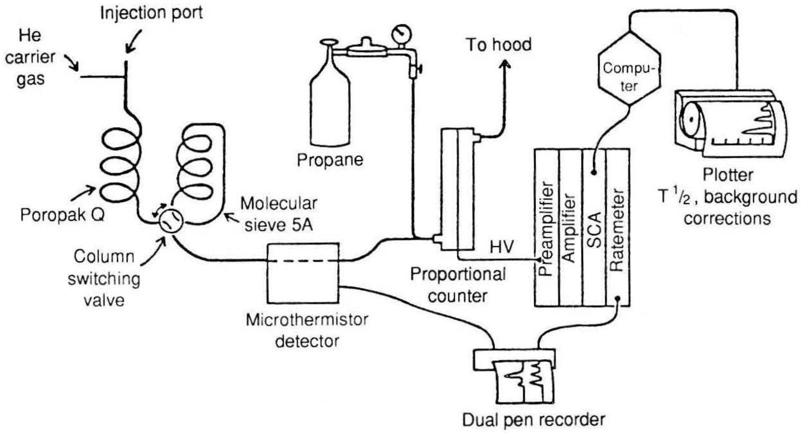


Figure 8 Schematic of the gas chromatography system using helium carrier gas coupled to proportional counters to monitor ^{13}N gaseous products of denitrification. After sample injection, ^{13}NO and $[^{13}N]N_2$ are held on the molecular sieve 5A column while $[^{13}N]N_2O$ elutes from the Porapak Q column; the carrier flow is then redirected to elute $[^{13}N]N_2$ followed by ^{13}NO. The microthermistor and proportional counter detect stable and radioactive N products, respectively. [Reproduced from Tiedje *et al.* (1979) *Soil Science of America Journal*, Volume 43, No. 4, 1979, pages 709–716, by permission of the Soil Science Society of America, Inc.]

analytical uses by Tiedje *et al.* (1979, 1981) and Chasko and Thayer (1981). In most analytical applications, the ions are best separated using a silica-based anion-exchange material (e.g., Partisil-10 SAX, Whatman) in an analytical column (4.6 mm ID × 25 cm length). Analytical columns are typically operated using 30–50 m*M* phosphate buffer, pH 2.9–3.0, as the liquid phase at flow rates of 1.5–3.0 ml/min; these flow rates result in column pressures ranging from 800 to 2000 psi. The column effluent can be passed through a ultraviolet detector and NO_3^- and NO_2^- ions monitored by absorbance at 190–210 nm (Thayer and Huffaker, 1981). Sequentially, or alternatively, the effluent is passed through a 10-cm coiled loop either placed in the well of a NaI(Tl) counter (Chasko and Thayer, 1981) or between two NaI(Tl) crystals connected by coincidence circuitry (Tiedje *et al.*, 1981). A typical separation of radioions in target water is shown in Fig. 5A. Target water is loaded directly onto the column; however, biological or environmental samples need to be clarified by centrifugation (microcentrifuge at maximum speed for 1 min) or filtration (0.22-μm pore size) to avoid plugging the column.

D. Organic Products

Two basic approaches have been successfully applied to separation of ^{13}N-labeled amino acids resulting from cellular metabolism: HPLC and electrophoresis.

1. High-Performance Liquid Chromatography

HPLC separation of ^{13}N-labeled metabolites from mammalian tissue was pioneered by Cooper *et al.* (1979), who used a strong anion-exchange column. Kim and Hollocher (1982) used both strong anion- and cation-exchange columns to analyze bacterial lysates. More recently, Zehr and Falkowski (1988) used *o*-pthalaldehyde (OPA) derivatization and a reverse-phase C-18 column in $^{13}NH_4^+$ assimilation studies with a marine diatom.

Samples are prepared by homogenization of tissue in 1% picric acid (Cooper *et al.*, 1979), mixing equal volumes (0.5 ml each) of microbial incubation suspension and 0.5 *M* $HClO_4$ (Kim and Hollocher, 1982), or filtration onto glass fiber or polycarbonate filter pads followed by lysis of the filtered cells in 1 ml boiling HPLC solvent (Zehr and Falkowski, 1988) or 1 *M* $HClO_4$ at room temperature (J. Thayer, unpublished results). The lysates are clarified by centrifugation for 1–2 min at maximal speed in a microcentrifuge and filtered (0.22-μm pore size) before injection of 20 to 100 μl onto the HPLC column.

The running buffer for analytical anion-exchange columns (Partisil-10 SAX) is 5 m*M* KH_2PO_4-HCl, pH 3.5, at a flow rate of 1.3 ml/min. An example of amino acid separation by anion-exchange HPLC is shown in Fig. 9. The drawback to anion exchange is the coelution of weakly acidic and neutral amino acids such as glutamine, asparagine, alanine, and glycine, all of which have been detected in microbial and plant lysates (Wolk *et al.*, 1976; Schubert and Coker, 1981). Cooper *et al.* (1979) collected the major anion HPLC fractions and reanalyzed them by standard ion-exchange liquid chromatography. Kim and Hollocher (1982) applied cation-exchange HPLC in attempts to separate neutral amino acids. They utilized an Aminex HP-C, 4-mm ID × 25-cm long column (BioRad Laboratories, Richmond, California), with 0.2 *M* (with respect to Na^+) sodium citrate–HCl, pH 3.5, pumped at 0.7 ml/min as the buffer. An example of the separation is shown in Fig. 10; glycine and alanine are separately resolved, but glutamine, serine, and asparagine coelute.

Separation of OPA-derivatized ^{13}N-labeled asparate, glutamate, and glutamine on reverse-phase columns can be achieved by isocratic elution with a solvent of 20% methanol and 80% 50 m*M* sodium acetate, pH 5.9, at a flow rate of 1 ml/min (Zehr and Falkowski, 1988). Other organic products were not observed in this particular study. More elaborate gradient

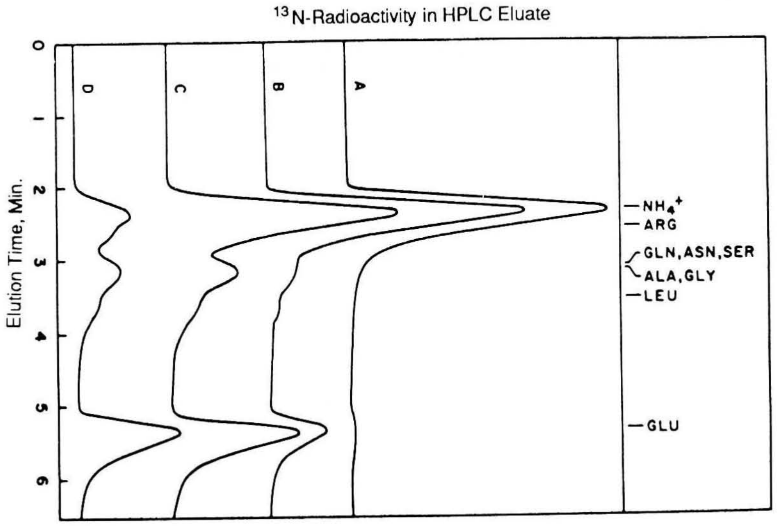


Figure 9 Anion-exchange HPLC radiochromatograms of $^{13}NH_4^+$ substrate and radiolabeled amino acids following assimilation by *Bacillus megaterium* for (A) 10 sec, (B) 70 sec, (C) 130 sec, and (D) 190 sec. Chromatography was on a Partisil-10 SAX column eluted with 5.0 m*M* potassium phosphate, pH 3.5. The column effluent was monitored with a NaI(Tl) well counter. [Reproduced, with permission, from Kim, C.-H., and Hollocher, T. C. (1982). ^{13}N isotope studies on the pathway of ammonia assimilation in *Bacillus megaterium* and *Escherichia coli. J. Bacteriol.* **151,** 358–366.]

elution of precolumn OPA-derivatized amino acids in reverse-phase HPLC is described by Cooper *et al.* (1984).

Radioactive peaks in HPLC separations can be monitored directly in the eluent using NaI(Tl) detectors, and the peak areas integrated and time-corrected. Alternatively, subsamples can be collected for analysis in a well counter.

2. Electrophoresis

Thin-layer electrophoretic separation of amino acids was adapted to ^{13}N analysis by Wolk *et al.* (1976). It has been applied to microbial (Meeks, 1981), plant (Skokut *et al.*, 1978; Meeks *et al.*, 1983), and mammalian (Thayer *et al.*, 1982) extracts. The keys to rapid separation are the use of high voltage (about 100 volts/cm; i.e., 2500–3000 volts total) and very thin layers (0.1 mm) of cellulose precoated on a 5-×-20-cm glass plate as the solid matrix (EM Sciences, No. 5772-3, or 5716-7, which can be cut to size).

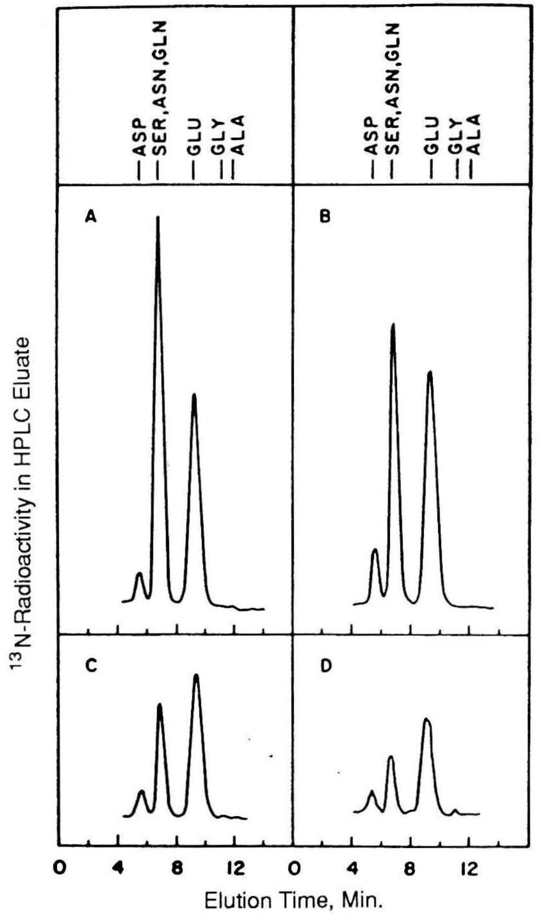


Figure 10 Cation exchange HPLC radiochromatograms of radiolabeled amino acids following assimilation of $^{13}NH_4^+$ by *Bacillus megaterium* for (A) 10 sec, (B) 70 sec, (C) 130 sec, and (D) 190 sec. Chromatography was on an Aminex HP-C column eluted with 0.2 *M* sodium citrate, pH 3.5, and the effluent monitored with a NaI(Tl) well counter. [Reproduced, with permission, from Kim, C.-H., and Hollocher, T. C. (1982). ^{13}N isotope studies on the pathway of ammonia assimilation in *Bacillus megaterium and Escherichia coli. J. Bacteriol.* **151,** 358–366.]

The ^{13}N assimilation reactions are stopped by mixing or homogenizing the samples in 80% methanol. The methanolic extracts are clarified by centrifugation (≥1000 × g for 1–2 min, depending on the tissue); the supernatant fluid is transferred to a 15-ml conical-bottom centrifuge tube and dried under vacuum at 50–55°C with manual agitation. The residue is dissolved in 0.2 ml of 80% methanol, with the sides of the tube thoroughly rinsed, and redried as before. The final residue is dissolved in 50 μl of 80% methanol to which is added 5 μl of a stock amino acid solution (1 mg each amino acid/ml). The suspension is then spotted on a 2-x-10-mm area parallel to the short axis of the 5-×-20-cm thin-layer plate; the spotting is done under a gentle stream of warm air from a hot air blower

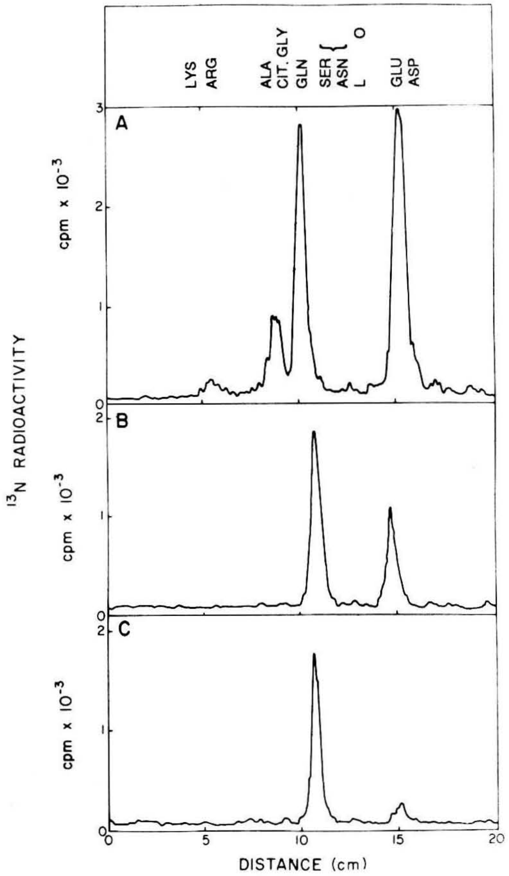


Figure 11 Radioelectrophoretograms of ^{13}N-labeled amino acids following assimilation of [^{13}N]N_2 by *Anabaena cylindrica* for (A) 120 sec, (B) 60 sec, and (C) 20 sec. Electrophoresis was on thin layers of cellulose in borate buffer, pH 9.2, for 12 min. The standard amino acids were visualized by spraying the thin-layer plates with acidic ninhydrin solution after scanning. [Reproduced, with permission, from Wolk, C. P., Thomas, J., Shaffer, P. W., Austin, S. M., and Galonsky, A. (1976). Pathway of nitrogen metabolism after fixation of ^{13}N-labeled nitrogen gas by the cyanobacterium, *Anabaena cylindrica*. *J. Biol. Chem.* **251**, 5027–5034.]

(or no air for analysis of $^{13}NH_4^+$; Meeks *et al.*, 1985a). A 10-μl Hamilton syringe with a needle point style 3 mounted in a 25-μl repeating dispenser is convenient for sample spotting. The lipid-soluble substances in the dried spotted material are displaced by ascending chromatography in the short axis of the plate in chloroform–methanol (varying ratios, 1:3 to 3:1 depending of the specific tissue). The plate is dried and sprayed with electrophoresis buffer, avoiding excess moisture; the buffer is generally 70 m*M* sodium borate, pH 9.2. The plate is connected to the buffer reservoirs by paper wicks (Whatman 3 mm) and electrophoresis is typically 8–10 min at 2500–3000 volts, with the thin-layer plate on a cooling paten and sandwiched between sheets of mylar (e.g., Model Q11 SAE 3202, Shandon Scientific Company, London). After electrophoresis, the distribution of ^{13}N on the thin-layer plate is determined with a radiochromatogram scanner (e.g., Model 7201, Packard Instruments Company) and the peak areas integrated and time-corrected. The standard amino acids are visualized after spraying the plates with 0.5% (w/v) ninhydrin made up in equal volumes of acetone and 0.35 *M* formic acid; the formic acid is necessary to neutralize the basic electrophoresis buffer.

An example of one-dimensional thin-layer electrophoretic separation and radioelectrophoretogram scanning is shown in Fig. 11. The disadvantage in one-dimensional electrophoretic separation is the close migration of aspartate and glutamate and of citrulline, alanine, and glycine. These amino acids can be resolved postelectrophoresis by ascending chromatography in the second dimension using phenol–water (3:1, v/v) as solvent and an atmosphere equilibrated with 3% (v/v) NH_4OH (Wolk *et al.*, 1976). The plate can be scanned in the second dimension by fragmentation into 5-×-5-cm sections (Wolk *et al.*, 1976) or by use of a two-dimensional scanner (Meeks, *et al.*, 1977; Markham *et al.*, 1979).

Acknowledgments

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